

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
14 November 2002 (14.11.2002)

PCT

(10) International Publication Number  
WO 02/090520 A2

(51) International Patent Classification <sup>7</sup> :	C12N	(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.
(21) International Application Number:	PCT/US02/14915	
(22) International Filing Date:	9 May 2002 (09.05.2002)	
(25) Filing Language:	English	
(26) Publication Language:	English	(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant: YALE UNIVERSITY [US/US]; 451 College Street, New Haven, CT 06520 (US).

## Published:

— without international search report and to be republished upon receipt of that report

(72) Inventors: MEDZHITOV, Ruslan; 310 Cedar Street, New Haven, CT 06520-8011 (US). HORNG, Tiffany; 100H Howe Street, Apt. 412, New Haven, CT 06511 (US). BARTON, Gregory; 62 Woodbine Street, Hamden, CT 06517 (US).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(74) Agents: HALLIDAY, Christopher, I. et al.; Morgan, Lewis &amp; Bockius LLP, 1701 Market Street, Philadelphia, PA 19103 (US).

(54) Title: TOLL/INTERLEUKIN-1 RECEPTOR ADAPTOR PROTEIN (TIRAP)

h TIRAP	1	M A S S T S L P A P G S R P K K P L G K M A D W F R O T I L L K K P K K R P N S P	40
m TIRAP	1	M A S S S S V P A S S T P S K K P R D K I A D W F R O A L L K K P K K M P I S O	40
h TIRAP	41	E S T S S D A S O P T S O D - S P - - - L P P S L S S V T S - - - - -	66
m TIRAP	41	E S H L Y D C S Q T A T Q D G L S P S S C S S P P S H S S P E S R S S P S S C S	80
h TIRAP	67	- - - - E S L P P T H A S D S G S S - - R W S K D Y D V C V C H S E E D L V A A	100
m TIRAP	81	S C M S P T S R P T H V D S S S S S G R W S K D Y D V C V G H S E E D L E A A	120
h TIRAP	101	C D L V S Y L E G S T A S I R C F I L O R D A T R G G A I V S E L C O A L S S	140
m TIRAP	121	Q E E V S Y L E G S O A S I R C F I L O R D A A P C G A I V S E L C Q A L S R S	160
h TIRAP	141	H C R V A L E I T P G F I E O D P W C K Y O M L Q A L T E A P G A E G C T I P L L S	180
m TIRAP	161	H C R V V L I T P G F I R D P W C K Y O M L Q A L T E A P A S E G C T I P L L S	200
h TIRAP	181	G L S R A A Y P P E E L R F M Y Y V D G R C P D G G F R O V K E A V M R C K L L Q	220
m TIRAP	201	G L S R A A Y P P E E L R F M Y Y V D G R G K D G G F Y D N K E A V I H Y L E T L	240
h TIRAP	221	E G E G E R D S A T V S D L L	221
m TIRAP	241	S	241

WO 02/090520 A2

(57) Abstract: TIRAP polypeptides and polynucleotides and methods for producing such polypeptides by recombinant techniques are disclosed. Also disclosed are methods for utilizing TIRAP polypeptides and polynucleotides in therapy, and diagnostic assays for such.

**Toll/Interleukin-1 Receptor Adapter Protein (TIRAP)****BACKGROUND OF THE INVENTION**

The drug discovery process is currently undergoing a fundamental revolution as it embraces "functional genomics," that is, high throughput genome- or gene-based biology. This approach is rapidly superceding earlier approaches based on positional cloning. A phenotype, that is a biological function or genetic disease, would be identified and this would then be tracked back to the responsible gene, based on its genetic map position.

10 Functional genomics relies heavily on the various tools of bioinformatics to identify gene sequences of potential interest from the many molecular biology databases now available. There is a continuing need to identify and characterize further genes and their related polypeptides/proteins, as targets for drug discovery with the potential for affecting immune response.

15 The strategy of innate immune recognition is based on the detection of constitutive and conserved products of microbial metabolism. Many metabolic pathways and individual gene products are unique to microorganisms and absent from host cells. Although these targets of recognition are not absolutely identical between different species of microbes, the gene products may be found in the 20 context of a common molecular pattern, which is typically highly conserved and invariant among microbes of a given class. Because the targets of innate immune recognition are conserved molecular patterns, they are called pathogen associated molecular patterns (PAMPs).

25 The recent discovery and characterization of the Toll-like receptor (TLR) family have incited new interest in the field of innate immunity. TLRs are pattern recognition receptors that have a unique and critical function in animal immunity. TLRs typically are transmembrane receptors characterized by an extracellular leucine rich repeats domain and an intracellular TIR (Toll/Interleukin-1 Receptor) domain. The TIR domain is a conserved protein-protein interaction 30 module and plays a role in host defense. In other words, TLRs play a critical role in microbial recognition and control of adaptive immune responses.

In mammalian species, there are at least ten (10) TLRs, and each has a distinct function in innate immune recognition. The TLRs mainly differ from one another with regard to ligand specificity, the use of accessory molecules, expression profiles and differences in signal transduction pathways.

5 Human TLR4 was the first identified and functionally characterized mammalian Toll. TLR4 functions as the signal transducing receptor for the PAMP lipopolysaccharide (LPS) as well as other PAMPs, which are apparent to one skilled in the art.

10 Activation of signal transduction pathways by TLRs leads to the induction of a variety of genes that function in host defense including inflammatory cytokines, chemokines, MHC and co-stimulatory molecules. Mammalian TLRs also induce multiple effector molecules such as inducible nitric oxide synthetase and antimicrobial peptides that can directly destroy microbial pathogens.

15 The signaling pathway, which appears to be shared by all members of the Toll and Interleukin-1 Receptor (IL-1R) families, includes four essential components: the adapters TRAF6, MyD88 and Tollip and a protein kinase, IRAK. MyD88 contains two protein interaction domains: an N-terminal death domain and a C-terminal TIR domain. The TIR domain of MyD88 associates with the TIR domain of TLR and the IL-1R, while the death domain interacts with the death 20 domain of IRAK.

25 In cells wherein MyD88 expression has been suppressed (i.e. in MyD88 knockout mice), macrophages and dendritic cells do not produce cytokines IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-12 when stimulated with LPS, MALP-2 or CpG, which signal through TLR4, TLR2 and TLR9, respectively. Consequently, MyD88 knockout mice are resistant to endotoxic shock. Furthermore, when normal bone marrow-derived dendritic cells (BMDCs) are stimulated with LPS or CpG, they produce large amounts of IL-12 and upregulate cell surface expression of MHC and co-stimulatory molecules. However, in MyD88 deficient BMDCs, stimulation with LPS or CpG does not produce IL12 or IL-6.

30 Additionally, RNA-dependent protein kinase (PKR)-deficient cells fail to activate c-Jun N-terminal Kinase (JNK) and p38 MAP Kinase (p38) in response to LPS stimulation. As TLR4 is required for signals downstream of LPS,

this indicates that PKR is a component of the TLR4 signaling pathway. Although phosphorylated PKR can be detected in LPS stimulated wild-type macrophages, phosphorylated PKR has also been detected in LPS stimulated MyD88-deficient macrophages. Interestingly, PKR from the MyD88-deficient macrophages was 5 activated with slower kinetics.

Although some cellular responses are completely abolished in MyD88-deficient cells, TLR4, but not TLR9 or TLR2, can still activate NF- $\kappa$ B and MAP kinases. This difference indicates that another adapter protein exists that can mediate MyD88-independent signaling in response to TLR4 ligation.

10 Thus, there is a need to determine the structure and function of the adapter protein involved in the MyD88 independent signaling downstream of TLR4 to develop compounds to treat diseases related to TLR4 function.

## SUMMARY OF THE INVENTION

15 The present invention relates to Toll Interleukin-1 Receptor Adapter Proteins (TIRAPs), and in particular TIRAP polypeptides and TIRAP polynucleotides, recombinant materials and methods for their production. In yet a further aspect, the present invention relates to TIRAP inhibitors. In another aspect, the invention relates to methods for using such polypeptides and polynucleotides, 20 including the treatment of inflammation and inducing or affecting immune response. In a further aspect, the invention relates to methods for identifying agonists and antagonists/inhibitors using the materials provided by the invention, and treating conditions associated with TIRAP imbalance with the identified compounds. In yet a further aspect, the invention relates to transgenic mammals 25 comprising TIRAP polynucleotides.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a sequence alignment of human and mouse TIRAP.

30 Figure 2, comprising Figures 2A through 2D, is a series of graphs which demonstrate that TIRAP is a component of the TLR4 signaling pathway, but not of the IL-1R signaling pathway. On the y axis, luciferase activity is expressed

as fold induction or relative light units (RLU). The x axis shows the transfected constructs: pcDNA3 (control vector); TIRAP (encodes full length TIRAP); TIRAP N (encodes N-terminal domain of TIRAP); TIRAP C (encodes C-terminal domain of TIRAP); TIRAP P125H (encodes TIRAP containing mutation at amino acid 5 125); CD4/TLR4 (encodes constitutively active form of CD4); MyD88 DN (encodes dominant negative mutant of MyD88); IL-1R/AcP (encodes IL-1 receptor and receptor accessory protein); TLR9 (encodes full length TLR9). Shaded triangles below the constructs indicate that increasing amounts of that construct was added.

10 Figure 3, comprising Figures 3A and 3B, is a pair of images of immunoblots demonstrating co-immunoprecipitation of TIRAP with TLR4 and MyD88. Cells were transfected in the presence (+) or absence (-) of constructs encoding a wild-type portion of TLR4 (TLR4 wt), a mutant derivative thereof (TLR4 mut), wild-type TIRAP (TIRAP), a mutant TIRAP containing a P125H 15 mutation (TIRAP PH), or wild-type MyD88 (MyD88), wherein each construct further encodes a Myc, Flag, or hemagglutinin (HA) tag, as indicated. Antibodies used for immunoprecipitation (IP) or immunoblot (IB) are indicated to the left of each blot.

20 Figure 4, comprising Figures 4A through 4C, is a series of images of immunoblots demonstrating that PKR co-immunoprecipitates with TIRAP and is a component of LPS and CpG-signaling pathways. (A) Bone marrow-derived macrophages from either wild-type (WT) or MyD88-deficient (MyD88<sup>-/-</sup>) mice were stimulated for the indicated time periods (minutes) with either LPS or CpG, and immunoblots were performed using an antibody that specifically recognizes 25 phosphorylated PKR (PKR-P). B and C Cells were transfected with constructs encoding Flag-tagged TIRAP and either a construct encoding an HA-tagged kinase dead mutant of PKR (HA PKR KD) or p58, as indicated. Antibodies used for immunoprecipitation (IP) or immunoblot (IB) are indicated to the left of each blot, and arrows on the right show the location of the indicated proteins.

30 Figure 5, comprising Figures 5A through 5D, is a series of blots depicting inhibition of LPS- but not CpG-induced NF- $\kappa$ B activation, PKR phosphorylation, and JNK phosphorylation by TIRAP. (A) TIRAP peptide, but not a reverse sequence peptide, inhibited LPS-induced NF- $\kappa$ B activation. RAW  $\kappa$ B

cells pretreated for 1h with either the TIRAP or control peptide were stimulated with LPS (10 ng/ml) for 5h. Samples were stimulated in duplicate. (B) Pretreatment of RAW cells with TIRAP peptide blocks LPS-induced I $\kappa$ B- $\alpha$  degradation. RAW cells pretreated with the TIRAP peptide as indicated were either 5 left unstimulated or stimulated with LPS (10 ng/ml) for the indicated time periods. Lysates (30  $\mu$ g/sample) were resolved by SDS-PAGE followed by immunoblotting with an anti- I $\kappa$ B- $\alpha$  antibody to assess I $\kappa$ B- $\alpha$  degradation. (C) TIRAP peptide inhibited LPS- but not CpG-induced NF- $\kappa$ B activity. RAW  $\kappa$ B cells pretreated with the TIRAP peptide as indicated were stimulated with either LPS or CpG and 10 harvested for reporter assay. (D) TIRAP peptide inhibited PKR and JNK phosphorylation induced by LPS but not CpG. RAW cells either untreated or pretreated with 40  $\mu$ M of the TIRAP peptide were stimulated with either LPS or CpG as indicated. Lysates (30  $\mu$ g/sample) were analyzed by SDS-PAGE followed by immunoblotting with antibodies that specifically recognize phosphorylated PKR 15 or phosphorylated JNK.

Figure 6, comprising Figure 6A through 6C, is a series of graphs indicating that TIRAP induces dendritic cell maturation. (A) Wild-type and MyD88<sup>-/-</sup> dendritic cells (DCs) were induced with LPS or CpG and analyzed for expression of the costimulatory molecules B7.1 and B7.1 using flow cytometry. 20 (B) Production of the cytokines, IL-12 and IL-6, was measured in cells that were untreated (none) or treated with LPS or CpG in the presence or absence of TIRAP peptide. (C) T cell proliferation was measured in wild-type DCs that were untreated (none), treated with LPS alone (LPS), or treated with LPS in the presence of increasing amounts of TIRAP peptide (LPS+TIRAP peptide). T cell 25 proliferation was measured by incorporation of  $^3$ H-thymidine into cells, expressed as cpm  $\times 10^3$ .

Figure 7 is a diagram depicting a proposed mechanism for the function of TIRAP and an inhibitory TIRAP peptide. The horizontal black line differentiates the extracellular space (above the line) from the intracellular space (below the line). TLR4, TIRAP, MyD88, and an inhibitory TIRAP peptide are shown schematically. Solid arrows indicate activation of the indicated proteins (NF- $\kappa$ B, AP-1, and p38), whereas a dotted line indicates that activation of these 30 proteins is inhibited.

Figure 8 graphically represents deficient B cell proliferation in cells from TIRAP knock-out mice which were exposed to 3H thymidine. The open square and black diamond denote the results from TIRAP knock-out mice K01 and K02, respectively, and the open triangle represents a wild-type (WT) control. As a measure of B cell proliferation, a beta radiation counter determined the amount 3H thymidine incorporated into B cells by counting the number of radioactive emissions over time.

Figure 9 graphically represents deficient cytokine production by dendritic cells from TIRAP knock-out mice. The black and striped bars denote the results from TIRAP knock-out mice K01 and K02, respectively, and the open bar represents a wild-type (WT) control. As a measure of cytokine production, B cells were stimulated with LPS and BLP and harvested for ELISA, using IL-6 and TNF- $\alpha$  capture antibodies and appropriate biotin-conjugated detection antibodies.

## 15 DESCRIPTION OF THE INVENTION

As described briefly above, some cellular responses are completely abolished in MyD88-deficient cells, yet TLR4, but not TLR9 or TLR2, can still activate NF- $\kappa$ B and MAP kinases. Specifically, a detailed analysis of the NF- $\kappa$ B and MAP signaling pathways demonstrates that the pathogen associated molecular pattern (PAMP) LPS, but not CpG or MALP-2, induces activation of NF- $\kappa$ B, JNK, p38 and ERK in MyD88 deficient cells. Activation of these signaling pathways occurred with delayed kinetics and was insufficient for the induction of cytokine gene expression.

Furthermore, MyD88 deficient BMDCs stimulated with LPS or CpG do not produce IL-12 or IL-6. However, BMDCs can be induced to upregulate expression of MHC and co-stimulatory molecules (such as CD80 and CD86), when treated with LPS, but not when stimulated with CpG. This result demonstrates that a MyD88 independent signaling pathway is sufficient for dendritic cell maturation, whereas the MyD88-dependent signaling pathway is required for the induction of cytokines IL-6 and IL-12. In addition, caspase-1 processing of Interleukin-18 into its biologically active form can be induced by TLR4, independently of MyD88.

These differences indicate that another adapter protein exists that can mediate MyD88-independent signaling in response to TLR4 ligation. As used herein, the adapter protein that mediates MyD88 independent signaling in response to TLR4 ligation will be referred to as "TIRAP" (Toll/Interleukin-1 Receptor Adapter Protein).

In particular, polypeptides of the present invention include isolated polypeptides encoded by the human and mouse polynucleotides comprising the sequences set forth in SEQ ID NO:1 and 2, respectively. Polypeptides of the present invention are believed to be members of the Toll/Interleukin-1 Receptor Adapter Protein family including MyD88. This gene is therefore of interest because it presents an alternative mechanism to induce or inhibit dendritic cell maturation as well as activation of NF- $\kappa$ B, JNK, p38 and ERK in cells.

As one of skill in the art understands, TLR4 is involved in numerous physiological responses, including infection and cell damage. For example, as indicated above TLR4 recognizes the PAMP LPS. TLR4 has also been implicated in inflammation responses, and in particular cellular necrosis induced inflammation. Accordingly, the discovery and characterization of molecules that affect and/or are involved in the signal pathways of TLR4 is important for the development of effective treatments for the above-described conditions as well as other conditions which will be appreciated by one of skill in the art.

#### **TIRAP and TIRAP Inhibitor Polynucleotides and Polypeptides Generally**

In a first aspect, the present invention relates to TIRAP polynucleotides and TIRAP polypeptides. Specifically, the polynucleotides set forth in SEQ ID NO:1 and 2 encode the polypeptides set forth in SEQ ID NO:3 (human) and 4 (mouse), respectively, which comprise the Toll Interleukin-1 Receptor Adapter Protein of the present invention. The human and mouse polynucleotides encoding TIRAP are highly homologous (See Figure 1). Specifically, in one embodiment, a comparison of human and mouse TIRAP polynucleotides and polypeptides indicates at least 73% identity at the protein level and 83% identity at the polynucleotide level. Thus it will be understood, based on the present disclosure, that both human and mouse TIRAP polynucleotides and the polypeptides encoded thereby can be used for similar purposes, as described herein.

The polynucleotides and polypeptides set forth in SEQ ID NOS: 1-4 are the full length TIRAP molecules and are hereinafter referred to generally as TIRAP or TIRAP polynucleotides, TIRAP peptides and TIRAP polypeptides. Unless otherwise specified, "TIRAP" is synonymous with "TIRAP polypeptide," and is 5 meant to refer to the Toll Interleukin-1 Receptor Adapter Protein and other polypeptides that have the same biological function, i.e., that modulate MyD88 independent signaling in response to their activation. The properties of the polypeptides set forth in SEQ ID NO:3 and 4 are also hereinafter referred to as "TIRAP activity" or "TIRAP polypeptide activity" or "biological activity of 10 TIRAP," as explained below.

Preferred polypeptides and polynucleotides of the present invention having homology to TIRAP polynucleotides and polypeptides as explained below, are expected to have similar biological functions/properties to TIRAP polypeptides and polynucleotides. Specifically, preferred TIRAP polypeptides of the present 15 invention have TIRAP activity in that they can bind to the TIRAP binding domain of TLR4. Such TIRAP polypeptides are therefore useful for drug screening, e.g., as described below. the TIRAP polypeptides of the invention also include such polypeptides that, upon activation of TLR4, signal a MyD88 independent response, e.g., activation of NF- $\kappa$ B and MAP kinases.

20 The characterization of the TIRAP polypeptide and the polynucleotide encoding the TIRAP polypeptide has led to the discovery of compounds that inhibit both the MyD88 independent pathway as well as the MyD88 dependent pathway of TLR4 by inhibiting TIRAP polypeptide mediated signaling. Such compounds that inhibit TIRAP activity are useful to prevent or 25 reduce inflammation and septic shock, and in particular to assist in the prevention of graft rejection, which occurs, at least in part, because of cellular necrosis induced inflammation.

Therefore, in a second aspect, the present invention relates to TIRAP 30 inhibitors. Specifically, the polypeptides set forth in SEQ ID NOS: 9 and 10 are human and mouse, respectively, TIRAP inhibitor polypeptides. These polypeptides are referred to herein as TIRAP inhibitors or TIRAP mutant peptides or TIRAP inhibitor polypeptides. The properties of each of the TIRAP inhibitors are hereinafter referred to as "TIRAP inhibitor activity." The polynucleotide set forth

in sequences SEQ ID NO: 5 (naturally occurring) and SEQ ID NO: 6 (degenerate) encode the human TIRAP inhibitor polypeptide set forth in SEQ ID NO: 9, and the polynucleotide set forth in sequences SEQ ID NO: 7 (naturally occurring) and SEQ ID NO: 8 (degenerate) encode the mouse TIRAP inhibitor polypeptide set forth in SEQ ID NO: 10. The SEQ ID NOS: 5-8 are referred to as TIRAP inhibitor polynucleotides and the sequences set forth in SEQ ID NOS: 9 and 10 are referred to as TIRAP inhibitor polypeptides.

Preferred inhibitor polypeptides and inhibitor polynucleotides of the present invention, having homology to TIRAP inhibitor polypeptides and polynucleotides as explained further below, are expected to have similar biological functions/properties to TIRAP inhibitor polypeptides and polynucleotides. As used herein, preferred TIRAP inhibitor polypeptides of the present invention have TIRAP inhibitor activity in that they can, notwithstanding activation of TLR4, inhibit both the MyD88 independent response, e.g., activation of NF- $\kappa$ B and MAP kinases and the MyD88 dependent response in cells expressing TLR4, as described in more detail below.

Lastly, SEQ ID NO: 11 is a functional example of a fusion protein of Antennapedia and a TIRAP inhibitor polypeptide. Specifically, SEQ ID NO: 11 is a fusion protein of Antennapedia and the TIRAP inhibitor set forth in SEQ ID NO: 10. Because the polypeptide set forth in SEQ ID NO: 11 also inhibits TIRAP activity, the properties of the TIRAP inhibitor fusion protein are also referred to as "TIRAP inhibitor activity." This particular embodiment is described in more detail below under the heading "Fusion Proteins."

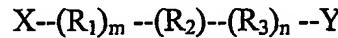
#### **The Structure of Polypeptides and Polynucleotides of the Present Invention**

The polypeptides of the present invention may be in the form of the "mature" protein or may be a part of a conjugated protein such as a fusion protein, e.g., SEQ ID NO: 11, as described below under "Fusion Proteins." It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production.

The present invention also includes variants of the aforementioned polypeptides, that is polypeptides that vary from the reference polypeptide by conservative amino acid substitutions, whereby a residue is substituted by another with like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acids are substituted, deleted, or added in any combination.

Such polypeptides include isolated polypeptides comprising an amino acid sequence which has at least 70% identity, preferably at least 80% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, most preferably at least 97-99% identity, to that of SEQ ID NOS: 3, 4, 9, 10 and 11 over the entire length of SEQ ID NOS: 3, 4, 9, 10 and 11. Such polypeptides include a polypeptide comprising the polypeptide set forth in SEQ ID NOS: 3, 4, 9, 10 and 11, as well as a polypeptide consisting of the polypeptide set forth in SEQ ID NO: 3, 4, 9, 10 and 11.

The invention also includes a polypeptide consisting of or comprising a polypeptide of the formula:



wherein, at the amino terminus, X is hydrogen, a metal or any other moiety described herein for modified polypeptides, and at the carboxyl terminus, Y is hydrogen, a metal or any other moiety for modified polypeptides, including the modifications described below, R<sub>1</sub> and R<sub>3</sub> are any amino acid residue or modified amino acid residue, m is an integer between 1 and 1000 or zero, n is an integer between 1 and 1000 or zero, and R<sub>2</sub> is an amino acid sequence of the invention or modified forms thereof, as described above. In the formula above, R<sub>2</sub> is oriented so that its amino terminal amino acid residue is at the left, covalently bound to R<sub>1</sub>, and its carboxy terminal amino acid residue is at the right, covalently bound to R<sub>3</sub>. Any stretch of amino acid residues denoted by either R<sub>1</sub> or R<sub>3</sub>, where m and/or n is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer. Other embodiments of the invention are provided where m is an

integer between 1 and 50, 100 or 500, and n is an integer between 1 and 50, 100, or 500.

5 Polypeptides of the present invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

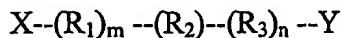
10 Polynucleotides of the present invention include isolated polynucleotides comprising a nucleotide sequence which has at least 70% identity, preferably at least 80% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, the polynucleotides set forth in SEQ ID NOS: 1 and 2 or the polynucleotides set forth in SEQ ID NOS: 5-8 over the entire length of one of SEQ ID NOS: 1, 2, and 5-8. In this regard, polynucleotides which have at least 97% identity are highly preferred, while those with at least 98-99% identity 15 are more highly preferred, and those with at least 99% identity are most highly preferred. Such polynucleotides include a polynucleotide comprising the polynucleotides set forth in SEQ ID NO: 1 and 2 as well as the polynucleotide consisting of the polynucleotides set forth in SEQ ID NO: 1 and 2. Such polynucleotides also include polynucleotides comprising the polynucleotides set 20 forth in SEQ ID NO: 5-8 as well as the polynucleotides consisting of the polynucleotides set forth in SEQ ID NO: 5-8.

25 As described above, the present invention also relates to both TIRAP polypeptide encoding polynucleotides and TIRAP inhibitor encoding polynucleotides. As one of skill in the art understands, a nucleotide sequence encoding one of the polypeptides set forth in SEQ ID NOS: 3, 4, 9, 10 may be identical to the polypeptide encoding sequence set forth in SEQ ID NOS: 1, 2, 5 and 7, respectively, or it may be a sequence other than the one set forth in SEQ ID NOS: 1, 2, 5, and 7 which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptide of SEQ ID NOS: 3, 4, 9, and 10.

30 Such polynucleotides include isolated polynucleotides comprising a nucleotide sequence encoding a polypeptide which has at least 70% identity, preferably at least 80% identity, more preferably at least 90% identity, yet more

preferably at least 95% identity, to the amino acid sequences of SEQ ID NOS: 3, 4, 9, 10 and 11, over the entire length of SEQ ID NOS: 3, 4, 9, 10 and 11. In this regard, polynucleotides encoding polypeptides which have at least 97% identity to the polypeptides set forth in SEQ ID NOS: 3, 4, 9, 10 and 11 are highly preferred, 5 while those that encode polypeptides with at least 98-99% identity to the polypeptides set forth in SEQ ID NOS: 3, 4, 9, 10 and 11 are more highly preferred, and those that encode polypeptides which have at least 99% identity to the polypeptides set forth in SEQ ID NOS: 3, 4, 9, 10 and 11 are most highly preferred.

10 The invention also includes a polynucleotide consisting of or comprising a polynucleotide of the formula:



wherein, at the 5' end of the molecule, X is hydrogen, a metal or a modified nucleotide residue, or together with Y defines a covalent bond, and at the 3' end of the molecule, Y is hydrogen, a metal, or a modified nucleotide residue, or 15 together with X defines the covalent bond, each occurrence of  $R_1$  and  $R_3$  is independently any nucleic acid residue or modified nucleic acid residue, m is an integer between 1 and 3000 or zero, n is an integer between 1 and 3000 or zero, and  $R_2$  is a nucleic acid sequence or modified nucleic acid sequence of the invention, as described herein. In the polynucleotide formula above,  $R_2$  is oriented so that its 5' 20 end nucleic acid residue is at the left, bound to  $R_1$ , and its 3' end nucleic acid residue is at the right, bound to  $R_3$ . Any stretch of nucleic acid residues denoted by either  $R_1$  and/or  $R_2$ , where m and/or n is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer. Where, in a particular embodiment, X and Y together define a covalent bond, the 25 polynucleotide of the above formula is a closed, circular polynucleotide, that can be a double-stranded polynucleotide wherein the formula shows a first strand to which the second strand is complementary. In another embodiment m and/or n is an integer between 1 and 1000. Other embodiments of the invention are provided where m is an integer between 1 and 50, 100 or 500, and n is an integer between 1 30 and 50, 100, or 500.

The invention also provides polynucleotides that are complementary to all the above-described polynucleotides.

**TIRAP Inhibitors**

As set forth above, SEQ ID NOS: 9-11 are TIRAP inhibitors. The polypeptides set forth in SEQ ID NOS: 9 and 10 are mutated forms of the binding domain of the polypeptides set forth in SEQ ID NO: 3 and 4, respectively, the 5 TIRAP polypeptide.

A typical TIRAP inhibitor may be 1) a small molecule mimetic or 2) a polypeptide encoded by the polynucleotides set forth in SEQ ID NO: 5-8, which are themselves mutated fragments of SEQ ID NO: 1 and 2, as described in more detail below or 3) another polypeptide having TIRAP inhibitor activity, as 10 described above.

Preferably, an effective TIRAP inhibitor polypeptide of the present invention has an encoded histidine to proline mutation at amino acid position 145 (as measured in mouse TIRAP, shown in Figure 1), or the proline to histidine mutation at amino acid position 125 (as measured in human TIRAP, also shown in 15 Figure 1 and referenced in the Figures as P125H) relative to the TIRAP polypeptide which does not have the encoded mutation. The mutation may be accomplished using techniques well understood to those of skill in the art.

It has been found that the histidine to proline mutation at the appropriate position enables a TIRAP inhibitor to bind to the cytoplasmic domain 20 of TLR4, yet inhibit downstream signaling of TLR4 (both MyD88 dependent and MyD88 independent signaling).

Without ascribing to a particular theory of cellular receptor signaling, it is believed that TIRAP inhibitors, as well as other polypeptides that are sufficiently homologous to TIRAP inhibitors or TIRAP inhibitor mimetics which 25 have TIRAP inhibitor activity, bind to TLR4. This binding is conceptually illustrated in Fig. 7. Once bound, the TIRAP inhibitor inhibits TLR4 signaling through both the MyD88 dependent pathway as well as the MyD88 independent pathway.

As suggested in the preceding paragraph, an organic compound, 30 typically with a molecular weight under 2 KD, having appropriate structural similarity to a TIRAP inhibitor polypeptide may be also used as a TIRAP inhibitor. For example, a small molecule mimetic may be used. As one of skill in the art

understands, small molecule mimetics are chemically synthesized compounds that provide the spatial conformation necessary to properly associate to a particular protein and elicit a response. Therefore, the present invention also comprises the use of a small molecule mimetic to inhibit TIRAP signaling.

## 5      **Fusion Proteins**

A further aspect of the invention also relates to polynucleotides encoding fusion proteins. Examples of fusion protein technology can be found in International Patent Application Nos. WO94/29458 and WO94/22914.

This particular embodiment of the present invention relates to fusion proteins comprising a TIRAP inhibitor polypeptide, or an active portion or active fragment thereof, and a cellular membrane transport protein, such as Antennapedia. As one of skill in the art understands, delivering proteins across the cell membrane so they become therapeutically active can be difficult. Viruses or virus fragments can be used to transport proteins into cells, but this technology is not universally approved. Antennapedia, obtained from the fruit fly *Drosophila*, is virtually identical to the human form of the protein and thus, there is little, if any, antigenic response in human use. Even though proteins can be transported using a part of Antennapedia (e.g., *Antp16*), such a method is typically only suitable for small proteins.

Although many embodiments of fusion proteins having TIRAP inhibitor activity are envisioned, one functional example of such an embodiment is set forth in SEQ ID NO: 11, which represents a fusion of Antennapedia and the TIRAP inhibitor set forth in SEQ ID NO: 10. It has been found that the TIRAP inhibitors set forth in SEQ ID NOS: 9 and 10 are of the appropriate size to be conjugated to Antennapedia and transported across cellular membranes. Specifically, once conjugated to a TIRAP inhibitor Antennapedia facilitates transport of the TIRAP inhibitor across the cellular membrane so that the polypeptide become therapeutically active within the cell and exhibits TIRAP inhibitor activity.

## 30     **Methods of Treating Abnormal Conditions**

In a further aspect, the present invention provides methods of treating abnormal conditions such as, for instance, inflammation, related to either an

excess of, or an under-expression of, TIRAP polypeptide activity. Preferably, the practice of this invention would comprise antagonizing or agonizing TIRAP activity to prevent or treat an inflammatory response.

If the activity of the TIRAP polypeptide is in excess, several 5 approaches are available. One approach comprises administering to a subject in need thereof a TIRAP inhibitor or TIRAP inhibitor mimetic (antagonist) as hereinabove described, optionally in combination with a pharmaceutically acceptable carrier, in an amount effective to inhibit the function of the polypeptide, such as, for example, by 1) binding an inhibitory molecule to TIRAP itself, thereby 10 inhibiting TIRAP function, or 2) by blocking the binding of TIRAP to TLR4 by binding a TIRAP inhibitor to TLR4 and thereby potentially inhibit both MyD88 dependent and independent signaling.

In still another approach, expression of the gene encoding an 15 endogenous TIRAP polypeptide can be inhibited using expression-blocking techniques. Known techniques involve the use of antisense sequences, either internally generated or separately administered (see, for example, O'Connor, J Neurochem (1991) 56:560 in Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, Fla. (1988)). Alternatively, oligonucleotides which form triple helices with the gene can be supplied (see, for 20 example, Lee et al., Nucleic Acids Res (1979) 6:3073; Cooney et al., Science (1988)241:456; Dervan et al., Science (1991)251:1360). These oligomers can be administered per se or the relevant oligomers can be expressed in vivo.

For treating abnormal conditions related to an under-expression of 25 TIRAP and its activity, several approaches are also available. One approach comprises administering to a subject a therapeutically effective amount of a compound which activates a polypeptide of the present invention, i.e., an agonist in combination with a pharmaceutically acceptable carrier (described below in the next section), to thereby alleviate the abnormal condition. Alternatively, gene therapy may be employed to effect the endogenous production of TIRAP by the 30 relevant cells in the subject. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct may then be isolated and introduced into a packaging cell transduced with a retroviral plasmid vector containing RNA

encoding a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a subject for engineering cells in vivo and expression of the polypeptide in vivo. For an overview of gene therapy, see Chapter 20, Gene 5 Therapy and other Molecular Genetic-based Therapeutic Approaches, (and references cited therein) in Human Molecular Genetics, T Strachan and A P Read, BIOS Scientific Publishers Ltd (1996). Another approach is to administer a therapeutic amount of a polypeptide of the present invention in combination with a suitable pharmaceutical carrier.

## 10 **Pharmaceutical Applications**

In a further aspect, the present invention provides for pharmaceutical compositions comprising a therapeutically effective amount of a polypeptide, such as the soluble form of a polypeptide of the present invention, agonist/antagonist peptide or small molecule compound, in combination with a pharmaceutically acceptable carrier or excipient. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned 15 compositions of the invention. Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as 20 therapeutic compounds.

The composition will be adapted to the route of administration, for instance by a systemic or an oral route. Preferred forms of systemic administration include injection, typically by intravenous injection. Other injection routes, such as 25 subcutaneous, intramuscular, or intraperitoneal, can be used. Alternative means for systemic administration include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if a polypeptide or other compounds of the present invention can be formulated in an enteric or an encapsulated formulation, oral administration may also be possible. 30 Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels, and the like.

The dosage range required depends on the choice of peptide or other compounds (such as a small molecule mimetic) of the present invention, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. Suitable dosages, 5 however, are typically in the range of 0.1-100  $\mu$ g/kg of subject. Wide variations in the needed dosage, however, are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these 10 dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

Polypeptides used in treatment can also be generated endogenously in the subject, in treatment modalities often referred to as "gene therapy" as described above. Thus, for example, cells from a subject may be engineered with a 15 polynucleotide, such as a DNA or RNA, to encode a polypeptide *ex vivo*, and for example, by the use of a retroviral plasmid vector. The cells are then introduced into the subject.

Polynucleotide and polypeptide sequences form a valuable information resource with which to identify further sequences of similar homology. 20 Storing the sequence in a computer readable medium and then using the stored data to search a sequence database using well known searching tools, such as GCC most easily facilitates this. Accordingly, in a further aspect, the present invention provides for a computer readable medium having stored thereon a polynucleotide comprising the sequence of SEQ ID NOS: 1, 2 and 5-8.

## 25 Methods of Production of Polypeptides and Polynucleotides

Polynucleotides of the present invention may be obtained, using standard cloning and screening techniques. Polynucleotides of the invention can also be obtained from natural sources such as genomic DNA libraries or can be synthesized using well known and commercially available techniques. Polypeptides 30 of the invention can also be synthesized using well known and commercially available techniques.

When polynucleotides of the present invention are used for the recombinant production of polypeptides of the present invention, the polynucleotide may include the coding sequence for the mature polypeptide, by itself, or the coding sequence for the mature polypeptide in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or preprotein sequence, or other fusion peptide portions. For example, a marker sequence, which facilitates purification of the fused polypeptide, can be encoded. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz et al., Proc Natl Acad Sci USA (1989) 86:821-824, or is an HA tag. The polynucleotide may also contain non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Further embodiments of the present invention include polynucleotides encoding polypeptide variants which comprise the amino acid sequence of SEQ ID NO:3 and 4 and in which several, for instance from 5 to 10, 1 to 5, 1 to 3, 1 to 2 or 1, amino acid residues are substituted, deleted or added, in any combination.

Polynucleotides which are identical or sufficiently identical to a nucleotide sequence contained in SEQ ID NO: 1, 2 and 5-8 may be used as hybridization probes for cDNA and genomic DNA or as primers for a nucleic acid amplification (PCR) reaction, to isolate full-length cDNAs and genomic clones encoding polypeptides of the present invention and to isolate cDNA and genomic clones of other genes (including genes encoding homologs and orthologs from species other than human) that have a high sequence similarity to SEQ ID NO: 1, 2 and 5-8. Typically these nucleotide sequences are 70% identical, preferably 80% identical, more preferably 90% identical, most preferably 95% identical to that of the referent. The probes or primers will generally comprise at least 15 nucleotides, preferably, at least 30 nucleotides and may have at least 50 nucleotides. Particularly preferred probes will have between 30 and 50 nucleotides.

A polynucleotide encoding a polypeptide of the present invention, including homologs and orthologs from species other than human, may be obtained by a process which comprises the steps of screening an appropriate library (as

understood by one of ordinary skill in the art) under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO:1, 2 or 5-8 or a fragment thereof; and isolating full-length cDNA and genomic clones containing said polynucleotide sequence. Such hybridization techniques are well known to the skilled artisan. Preferred stringent hybridization conditions include overnight incubation at 42 degrees Celsius in a solution comprising: 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 microgram/ml denatured, sheared salmon sperm DNA; followed by washing the filters in 0.1 x SSC at about 65 degree C. Thus the present invention also includes polynucleotides obtainable by screening an appropriate library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO: 1, 2 or 5-8 or a fragment thereof.

The skilled artisan will appreciate that, in many cases, an isolated cDNA sequence will be incomplete, in that the region coding for the polypeptide is cut short at the 5' end of the cDNA. This is a consequence of reverse transcriptase, an enzyme with inherently low processivity (a measure of the ability of the enzyme to remain attached to the template during the polymerization reaction), failing to complete a DNA copy of the mRNA template during 1st strand cDNA synthesis.

There are several methods available and well known to those skilled in the art to obtain full-length cDNAs, or extend short cDNAs, for example those based on the method of Rapid Amplification of cDNA ends (RACE) (see, for example, Frohman et al., PNAS USA 85, 8998-9002, 1988). Recent modifications of the technique, exemplified by the Marathon ® technology (Clontech Laboratories Inc.) for example, have significantly simplified the search for longer cDNAs. In the Marathon ® technology, cDNAs have been prepared from mRNA extracted from a chosen tissue and an 'adapter' sequence ligated onto each end. Nucleic acid amplification (PCR) is then carried out to amplify the 'missing' 5' end of the cDNA using a combination of gene specific and adapter specific oligonucleotide primers. The PCR reaction is then repeated using 'nested' primers, that is, primers designed to anneal within the amplified product (typically an adapter specific primer that anneals further 3' in the adapter sequence and a gene specific primer that anneals further 5' in the known gene sequence). The products of this reaction can then be analyzed by DNA sequencing and a full-length cDNA

constructed either by joining the product directly to the existing cDNA to give a complete sequence, or carrying out a separate full-length PCR using the new sequence information for the design of the 5' primer.

Recombinant polypeptides of the present invention may be prepared 5 by processes well known in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to expression systems which comprise a polynucleotide or polynucleotides of the present invention, to host cells which are genetically engineered with such expression systems and to the production of polypeptides of 10 the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs 15 of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the 15 present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis et al., Basic Methods in Molecular Biology (1986) and Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). Preferred such methods include, for instance, calcium 20 phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as streptococci, staphylococci, *E. coli*, *Streptomyces* and *Bacillus subtilis* 25 cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila S2* and *Spodoptera Sf9* cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used, for instance, chromosomal, episomal and virus-derived systems, e.g., vectors derived from 30 bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl

pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector which is able to maintain, propagate or express a polynucleotide to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook et al., MOLECULAR CLONING, A LABORATORY MANUAL (supra). Appropriate secretion signals may be incorporated into the desired polypeptide to allow secretion of the translated protein into the lumen of the endoplasmic reticulum, the periplasmic space or the extracellular environment. These signals may be endogenous to the polypeptide or they may be heterologous signals.

If a polypeptide of the present invention is to be expressed for use in screening assays (described below), the peptide may be produced intracellularly or secreted into the medium. If produced intracellularly, the cells must first be lysed before the polypeptide is recovered. The peptide may be membrane bound, for example, it may be fused with the cytoplasmic domain of a membrane protein, or it may contain sequence motifs which will undergo a lipid modification, such as palmitoylation, that will target the peptide to the plasma membrane. If the polypeptide is secreted into the medium, the medium can be recovered in order to recover and purify the polypeptide.

Polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well-known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

## Screening Techniques for TIRAP and TIRAP Inhibitors

As described above, TIRAP plays a role in many biological functions, including many disease states, in particular inflammation, septic shock and necrosis induced inflammation. It is therefore desirous to devise screening methods to identify compounds which stimulate or which inhibit the function of the TIRAP polypeptide. Accordingly, in a further aspect, the present invention provides for a method of screening compounds to identify those which stimulate or which inhibit the function of TIRAP. In general, agonists or antagonists may be employed for therapeutic and prophylactic purposes (described above).

10 Appropriate compounds may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures.

The screening method may simply measure the binding of a candidate compound to a polypeptide of the present invention, or a small molecule mimetic, or to cells or membranes bearing a polypeptide of the present invention, or 15 a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound. Alternatively, the screening method may involve competition with a labeled competitor. Further, these screening methods may test whether the candidate compound results in a signal generated by activation or inhibition of the polypeptide, using detection systems appropriate to the cells bearing the 20 polypeptide.

Inhibitors of activation are generally assayed in the presence of a known agonist and the effect on activation by the agonist by the presence of the candidate compound is observed. Constitutively active polypeptides may be employed in screening methods for inverse agonists or inhibitors, in the absence of 25 an agonist or inhibitor, by testing whether the candidate compound results in inhibition of activation of the polypeptide. Further, the screening methods may simply comprise the steps of mixing a candidate compound with a solution containing a polypeptide of the present invention, to form a mixture, measuring TIRAP activity in the mixture, and comparing TIRAP activity of the mixture to a 30 standard. In other words, the screening method comprises the steps of providing a polypeptide comprising at least the TIRAP-binding domain of TLR4, a TIRAP polypeptide or other polypeptide that comprises a TLR4 binding domain, and a candidate compound, placing the candidate compound into proximity with the

polypeptide comprising the TIRAP-binding domain of TLR4 and the TIRAP polypeptide or other polypeptide, and determining whether the candidate compound competes with the binding of either of the polypeptide comprising the TIRAP-binding domain of TLR4 or the TIRAP polypeptide or other polypeptide.

5 As one skilled in the art understands, fusion proteins, such as those made from a Fc portion of a polypeptide and a TIRAP polypeptide can also be used for high-throughput screening assays to identify antagonists for the polypeptide of the present invention (see D. Bennett et al., J Mol Recognition, 8:52-58 (1995); and K. Johanson et al., J. Biol Chem, 270:9459-9471 (1995)).

10 Examples of potential polypeptide antagonists include antibodies or, in some cases, oligonucleotides or proteins which are closely related to the ligands, substrates, receptors, enzymes, etc., as the case may be, of the polypeptide, e.g., a fragment of the ligands, substrates, receptors, enzymes, etc.; or small molecules which bind to the polypeptide of the present invention but do not elicit a response, 15 so that the activity of the polypeptide is prevented.

The polynucleotides, polypeptides and antibodies of the present invention may also be used to configure screening methods for detecting the effect of added compounds on the production of mRNA and polypeptide in cells. For example, an ELISA assay may be constructed for measuring secreted or cell 20 associated levels of polypeptide using monoclonal and polyclonal antibodies by standard methods known in the art. This can be used to discover agents which may inhibit or enhance the production of polypeptide from suitably manipulated cells or tissues.

Thus, in another aspect, the present invention relates to a screening 25 kit for identifying agonists, antagonists, other ligands and receptors, substrates, enzymes, etc. for polypeptides of the present invention; or compounds which decrease or enhance the production of such polypeptides, which comprises:  
(a) a polypeptide of the present invention;  
(b) a recombinant cell expressing a polypeptide of the present invention;  
30 (c) a cell membrane expressing a polypeptide of the present invention; or  
(d) antibody to a polypeptide of the present invention;  
which polypeptide is preferably that of SEQ ID NO:3 or 4.

It will be appreciated that in any such kit, (a), (b), (c) or (d) may comprise a substantial component.

It will be readily appreciated by the skilled artisan that a polypeptide of the present invention may also be used in a method for the structure-based design

5 of an agonist, antagonist or inhibitor of the polypeptide, by:

(a) determining in the first instance the three-dimensional structure of the polypeptide;

(b) deducing the three-dimensional structure for the likely reactive or binding site(s) of an agonist, antagonist or inhibitor;

10 (c) synthesizing candidate compounds that are predicted to bind to or react with the deduced binding or reactive site; and

(d) testing whether the candidate compounds are indeed agonists, antagonists or inhibitors.

15 It will be further appreciated that this will normally be an iterative process.

### **Antigenic Properties of the Polynucleotides and Polypeptides of the Present Invention**

The polypeptides of the invention or their fragments or analogs thereof, or cells expressing them, can also be used as immunogens to produce 20 antibodies immunospecific for polypeptides of the present invention. The term "immunospecific" means that the antibodies have substantially greater affinity for the polypeptides of the invention than their affinity for other related polypeptides in the prior art.

25 Antibodies generated against polypeptides of the present invention may be obtained by administering the polypeptides or epitope-bearing fragments, analogs or cells to an animal, preferably a non-human animal, using routine protocols. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., *Nature* (1975) 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., *Immunology Today* (1983) 4:72) and the EBV-hybridoma technique (Cole et al.,

MONOCLONAL ANTIBODIES AND CANCER THERAPY, pp. 77-96, Alan R. Liss, Inc., 1985).

Techniques for the production of single chain antibodies, such as those described in U.S. Pat. No. 4,946,778, can also be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms, including other mammals, may be used to express humanized antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography.

In a further aspect, the present invention relates to genetically engineered soluble fusion proteins comprising a polypeptide of the present invention, or a fragment thereof, and various portions of the constant regions of heavy or light chains of immunoglobulins of various subclasses (IgG, IgM, IgA, IgE). Preferred as an immunoglobulin is the constant part of the heavy chain of human IgG, particularly IgG1, where fusion takes place at the hinge region. In a particular embodiment, the Fc part can be removed simply by incorporation of a cleavage sequence which can be cleaved with blood clotting factor Xa. Furthermore, this invention relates to processes for the preparation of these fusion proteins by genetic engineering, and to the use thereof for, for example, drug screening.

### **Transgenic Cells and Mammals**

The nucleic and amino acids of the invention can be used to produce recombinant cells and transgenic non-human mammals which are useful tools for the study of TIRAP function. That is, the exogenous nucleic acid, or transgene as it is also referred to herein, of the invention is introduced into a cell, and the cell is then used to generate the non-human transgenic mammal. The cell into which the transgene is introduced is preferably an embryonic stem (ES) cell. However, the invention should not be construed to be limited solely to ES cells comprising the transgene of the invention nor to cells used to produce transgenic animals. Rather, a transgenic cell of the invention includes, but is not limited to, any cell derived from a transgenic animal comprising a transgene, a cell comprising the transgene

derived from a chimeric animal derived from the transgenic ES cell, and any other comprising the transgene which may or may not be used to generate a non-human transgenic mammal.

Further, it is important to note that the purpose of transgene-  
5 comprising, *i.e.*, recombinant, cells should not be construed to be limited to the generation of transgenic mammals. Rather, the invention should be construed to include any cell type into which a nucleic acid encoding TIRAP is introduced, including, without limitation, a prokaryotic cell and a eukaryotic cell comprising an isolated nucleic acid encoding TIRAP.

10 When the cell is a eukaryotic cell, the cell may be any eukaryotic cell which, when the transgene of the invention is introduced therein, and the protein encoded by the desired gene is no longer expressed therefrom, a benefit is obtained. Such a benefit may include the fact that there has been provided a system in which lack of expression of the desired gene can be studied *in vitro* in the  
15 laboratory or in a mammal in which the cell resides, a system wherein cells comprising the introduced gene deletion can be used as research, diagnostic and therapeutic tools, and a system wherein animal models are generated which are useful for the development of new diagnostic and therapeutic tools for selected disease states in a mammal.

20 Alternatively, the invention includes a eukaryotic cell which, when the transgene of the invention is introduced therein, and the protein encoded by the desired gene is expressed therefrom where it was not previously present or expressed in the cell or where it is now expressed at a level or under circumstances different than that before the transgene was introduced, a benefit is obtained. Such  
25 a benefit may include the fact that there has been provided a system in the expression of the desired gene can be studied *in vitro* in the laboratory or in a mammal in which the cell resides, a system wherein cells comprising the introduced gene can be used as research, diagnostic and therapeutic tools, and a system wherein animal models are generated which are useful for the development of new  
30 diagnostic and therapeutic tools for selected disease states in a mammal.

Such cell expressing an isolated nucleic acid encoding TIRAP can be used to provide TIRAP to a cell, tissue, or whole animal where a higher level of TIRAP can be useful to treat or alleviate a disease, disorder or condition associated with low level of TIRAP expression and/or activity. Such diseases, disorders or

conditions can include, but are not limited to, intestinal tumors, familial adenomatous polyposis, colon cancer, irritable bowel disease, inflammatory bowel disease, diabetes, obesity, tongue cancer, breast cancer, lung cancer, and the like. Therefore, the invention includes a cell expressing TIRAP to increase or induce 5 TIRAP expression, translation, and/or activity, where increasing TIRAP expression, protein level, and/or activity can be useful to treat or alleviate a disease, disorder or condition.

One of ordinary skill would appreciate, based upon the disclosure provided herein, that a "knock-in" or "knock-out" vector of the invention comprises 10 at least two sequences homologous to two portions of the nucleic acid which is to be replaced or deleted, respectively. The two sequences are homologous with sequences that flank the gene; that is, one sequence is homologous with a region at or near the 5' portion of the coding sequence of the nucleic acid encoding TIRAP and the other sequence is further downstream from the first. One skilled in the art 15 would appreciate, based upon the disclosure provided herein, that the present invention is not limited to any specific flanking nucleic acid sequences. Instead, the targeting vector may comprise two sequences which remove some or all (*i.e.*, a "knock-out" vector) or which insert (*i.e.*, a "knock-in" vector) a nucleic acid encoding TIRAP, or a fragment thereof, from or into a mammalian genome, respectively. The crucial feature of the targeting vector is that it comprise sufficient 20 portions of two sequences located towards opposite, *i.e.*, 5' and 3', ends of the TIRAP open reading frame (ORF) in the case of a "knock-out" vector, to allow deletion/insertion by homologous recombination to occur such that all or a portion of the nucleic acid encoding TIRAP is deleted from or inserted into a location on a 25 mammalian chromosome.

The design of transgenes and knock-in and knock-out targeting vectors is well-known in the art and is described in standard treatises such as Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in 30 Molecular Biology, John Wiley & Sons, New York), and the like. The upstream and downstream portions flanking or within the TIRAP coding region to be used in the targeting vector may be easily selected based upon known methods and following the teachings disclosed herein based on the disclosure provided herein including the nucleic and amino acid sequences of both mouse and human TIRAP.

Armed with these sequences, one of ordinary skill in the art would be able to construct the transgenes and knock-out vectors of the invention.

The invention further includes a knock-out targeting vector comprising a nucleic acid encoding a selectable marker such as, for example, a nucleic acid encoding the *neo*<sup>R</sup> gene thereby allowing the selection of transgenic a cell where the nucleic acid encoding TIRAP, or a portion thereof, has been deleted and replaced with the neomycin resistance gene by the cell's ability to grow in the presence of G418. However, the present invention should not be construed to be limited to neomycin resistance as a selectable marker. Rather, other selectable markers well-known in the art may be used in the knock-out targeting vector to allow selection of recombinant cells where the TIRAP gene has been deleted and/or inactivated and replaced by the nucleic acid encoding the selectable marker of choice. Methods of selecting and incorporating a selectable marker into a vector are well-known in the art and are described in, for example, Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York).

As noted herein, the invention includes a non-human transgenic mammal comprising an exogenous nucleic acid inserted into a desired site in the genome thereof thereby deleting the coding region of a desired endogenous target gene, *i.e.*, a knock-out transgenic mammal. Further, the invention includes a transgenic non-human mammal wherein an exogenous nucleic acid encoding TIRAP is inserted into a site in the genome, *i.e.*, a "knock-in" transgenic mammal. The knock-in transgene inserted may comprise various nucleic acids encoding, for example, a tag polypeptide, a promoter/regulatory region operably linked to the nucleic acid encoding TIRAP not normally present in the cell or not typically operably linked to TIRAP.

The generation of the non-human transgenic mammal of the invention is preferably accomplished using the method which is now described. However, the invention should in no way be construed as being limited solely to the use of this method, in that, other methods can be used to generate the desired knock-out mammal.

In the preferred method of generating a non-human transgenic mammal, ES cells are generated comprising the transgene of the invention and the

cells are then used to generate the knock-out animal essentially as described in Nagy and Rossant (1993, In: Gene Targeting, A Practical Approach, pp. 146-179, Joyner ed., IRL Press). ES cells behave as normal embryonic cells if they are returned to the embryonic environment by injection into a host blastocyst or 5 aggregate with blastomere stage embryos. When so returned, the cells have the full potential to develop along all lineages of the embryo. Thus, it is possible, to obtain ES cells, introduce a desired DNA therein, and then return the cell to the embryonic environment for development into mature mammalian cells, wherein the desired DNA may be expressed.

10 Precise protocols for the generation of transgenic mice are disclosed in Nagy and Rossant (1993, In: Gene Targeting, A Practical Approach, Joyner ed. IRL Press, pp. 146-179). and are therefore not repeated herein. Transfection or transduction of ES cells in order to introduce the desired DNA therein is accomplished using standard protocols, such as those described, for example, in 15 Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York). Preferably, the desired DNA contained within the transgene of the invention is electroporated into ES cells, and the cells are propagated as described in Soriano et al. (1991, Cell 64:693-702).

20 Introduction of an isolated nucleic acid into the fertilized egg of the mammal is accomplished by any number of standard techniques in transgenic technology (Hogan et al., 1986, Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor, NY). Most commonly, the nucleic acid is introduced into the embryo by way of microinjection.

25 Once the nucleic acid is introduced into the egg, the egg is incubated for a short period of time and is then transferred into a pseudopregnant mammal of the same species from which the egg was obtained as described, for example, in Hogan et al. (1986, Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor, NY). Typically, many eggs are injected per experiment, and 30 approximately two-thirds of the eggs survive the procedure. About twenty viable eggs are then transferred into pseudopregnant animals, and usually four to ten of the viable eggs so transferred will develop into live pups.

Any mammalian TIRAP gene may be used in the methods described herein to produce a transgenic mammal or a transgenic cell harboring a transgene

comprising a deletion of all or part of that TIRAP gene. Preferably, human TIRAP (SEQ ID NO:1) or mouse TIRAP (SEQ ID NO: 2) is also used.

The transgenic mammal of the invention can be any species of mammal. Thus, the invention should be construed to include generation of 5 transgenic mammals encoding the chimeric nucleic acid, which mammals include mice, hamsters, rats, rabbits, pigs, sheep and cattle. The methods described herein for generation of transgenic mice can be analogously applied using any mammalian species. Preferably, the transgenic mammal of the invention is a rodent and even more preferably, the transgenic mammal of the invention is a mouse. By way of 10 example, Lukkarinen et al. (1997, Stroke 28:639-645), teaches that gene constructs which enable the generation of transgenic mice also enable the generation of other transgenic rodents, including rats. Similarly, nullizygous mutations in a genetic locus of an animal of one species can be replicated in an animal of another species having a genetic locus highly homologous to the first species.

15 To identify the transgenic mammals of the invention, pups are examined for the presence of the isolated nucleic acid using standard technology such as Southern blot hybridization, PCR, and/or RT-PCR. Expression of the nucleic acid in the cells and in the tissues of the mammal is also assessed using ordinary technology described herein. Further, the presence or absence of TIRAP 20 the circulating blood of the transgenic animal can be determined, for example, as disclosed herein (e.g., Western blot analysis), or using standard methods for protein detection that are well-known in the art.

Cells obtained from the transgenic mammal of the invention, which are also considered "transgenic cells" as the term is used herein, encompass such as 25 cells as those obtained from the TIRAP (+/-) and (-/-) transgenic non-human mammal described elsewhere herein, are useful systems for modeling diseases and symptoms of mammals which are believed to be associated with altered levels of TIRAP expression such as colon cancer, familial adenomatous polyposis, irritable bowel disease, inflammatory bowel disease, intestinal tumors, breast cancer, tongue 30 cancer, diabetes, and obesity, and any other disease, disorder or condition associated with an altered level of TIRAP expression. Moreover, as a marker of a pathway(s) associated with tumor proliferation and other intestinal abnormalities such colon cancer, familial adenomatous polyposis, irritable bowel disease, inflammatory bowel disease, intestinal tumors, breast cancer, tongue cancer,

diabetes, and obesity, TIRAP expression levels are also useful indicators in assessment of such diseases, disorders or conditions.

Particularly suitable are cells derived from a tissue of the non-human knock-out or knock-in transgenic mammal described herein, wherein the transgene comprising the TIRAP gene is expressed or inhibits expression of TIRAP in various tissues. By way of example, cell types from which such cells are derived include fibroblasts, endothelial, adipocyte, and myoblast cells of (1) the TIRAP (+/+)<sup>5</sup>, (+/-) and (-/-) non-human transgenic liveborn mammal, (2) the TIRAP (+/+)<sup>10</sup>, (-/-) or (+/-) fetal animal, and (3) placental cell lines obtained from the TIRAP (+/+)<sup>10</sup>, (-/-) and (+/-) fetus and liveborn mammal.

The following definitions are provided to facilitate understanding of certain terms used frequently hereinbefore.

"Antibodies" as used herein includes polyclonal and monoclonal 15 antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library.

"Isolated" means altered "by the hand of man" from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or 20 removed from its original environment; or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

"Polynucleotide" generally refers to any polyribonucleotide or 25 polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded 30 or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term "polynucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with

backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. Polypeptides include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications may occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present to the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenylation, sulfation, transfer-RNA mediated addition

of amino acids to proteins such as arginylation, and ubiquitination (see, for instance, PROTEINS—STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; Wold, F., Post-translational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in 5 POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al., "Analysis for protein modifications and nonprotein cofactors", Meth Enzymol (1990) 182:626-646 and Rattan et al., "Protein Synthesis: Post-translational Modifications and Aging", Ann NY Acad Sci (1992) 663:48-62).

10 "Variant" refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference 15 polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are 20 closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic 25 variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

30 "Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as the case may be, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" can be readily calculated by known methods, including but not limited to

those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, 5 New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, 30 H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested.

10 Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S. F. et al., J Molec. Biol. 215: 403-410 (1990)).

15 The BLAST X program is publicly available from NCBI and other sources (BLASTManual, Altschul, S., et al, NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The well-known Smith Waterman algorithm may also be used to determine identity.

Parameters for polypeptide sequence comparison include the 20 following:

1) Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)  
Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992)  
Gap Penalty: 12

25 Gap Length Penalty: 4  
A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison Wis. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

30 Parameters for polynucleotide comparison include the following:  
1) Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)  
Comparison matrix: matches=+10, mismatch=0  
Gap Penalty: 50

Gap Length Penalty: 3

Available as: The "gap" program from Genetics Computer Group, Madison Wis.

These are the default parameters for nucleic acid comparisons.

A preferred meaning for "identity" for polynucleotides and  
5 polypeptides, as the case may be, are provided in (1) and (2) below.

(1) Polynucleotide embodiments further include an isolated polynucleotide comprising a polynucleotide sequence having at least a 50, 60, 70, 80, 85, 90, 95, 97 or 100% identity to the reference sequences of SEQ ID NO:1 or 2, wherein said polynucleotide sequence may be identical to the reference sequence 10 of SEQ ID NO:1 or 2 or may include up to a certain integer number of nucleotide alterations as compared to the reference sequence, wherein said alterations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or 15 anywhere between those terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence, and wherein said number of nucleotide alterations is determined by multiplying the total number of nucleotides in SEQ ID NO:1 or 2 by the integer defining the percent identity divided by 100 and then subtracting that 20 product from said total number of nucleotides in SEQ ID NO:1, or:

$$N_n = X_n - (X_n * y),$$

wherein  $N_n$  is the number of nucleotide alterations,  $X_n$  is the total number of nucleotides in SEQ ID NO:1 or 2,  $y$  is 0.50 for 50%, 0.60 for 60%, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%, 0.97 for 97% or 1.00 for 25 100%, and \* is the symbol for the multiplication operator, and wherein any non-integer product of  $X_n$  and  $y$  is rounded down to the nearest integer prior to subtracting it from  $X_n$ . Alterations of a polynucleotide sequence encoding the polypeptide of SEQ ID NO:3 or 4 may create nonsense, missense or frameshift 30 mutations in this coding sequence and thereby alter the polypeptide encoded by the polynucleotide following such alterations.

By way of example, a polynucleotide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:3 or 4, that is it

may be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the percent identity is less than 100% identity. Such alterations are selected from the group consisting of at least one nucleic acid deletion, substitution, including transition and

5 transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference polynucleotide sequence or anywhere between those terminal positions, interspersed either individually among the nucleic acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of nucleic acid alterations for a given percent identity is

10 determined by multiplying the total number of amino acids in SEQ ID NO:3 or 4 by the integer defining the percent identity divided by 100 and then subtracting that product from said total number of amino acids in SEQ ID NO: 3 or 4, or:

$$N_n = X_n - (X_n * y),$$

wherein  $N_n$  is the number of amino acid alterations,  $X_n$  is the total number of amino acids in SEQ ID NO:3 or 4,  $y$  is, for instance 0.70 for 70%, 0.80 for 80%, 0.85 for 85% etc., \* is the symbol for the multiplication operator, and wherein any non-integer product of  $X_n$  and  $y$  is rounded down to the nearest integer prior to subtracting it from  $X_n$ .

(2) Polypeptide embodiments further include an isolated polypeptide comprising a polypeptide having at least a 50, 60, 70, 80, 85, 90, 95, 97 or 100% identity to a polypeptide reference sequence of SEQ ID NO:3 or 4, wherein said polypeptide sequence may be identical to the reference sequence of SEQ ID NO:3 or 4 or may include up to a certain integer number of amino acid alterations as compared to the reference sequence, wherein said alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence, and wherein said number of amino acid alterations is determined by multiplying the total number of amino acids in SEQ ID NO:3 or 4 by the integer defining the percent identity divided by 100

and then subtracting that product from said total number of amino acids in SEQ ID

NO:3 or 4, or:

$$N_a = X_a - (X_a * y),$$

wherein  $N_a$  is the number of amino acid alterations,  $X_a$  is the total number of amino

5 acids in SEQ ID NO:3 or 4,  $y$  is 0.50 for 50%, 0.60 for 60%, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%, 0.97 for 97% or 1.00 for 100%, and \* is the symbol for the multiplication operator, and wherein any non-integer product of  $X_a$  and  $y$  is rounded down to the nearest integer prior to subtracting it from  $X_a$ .

10 By way of example, a polypeptide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:3 or 4, that is it may be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the percent identity is less than 100% identity. Such alterations are selected from the group consisting of 15 at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups 20 within the reference sequence. The number of amino acid alterations for a given % identity is determined by multiplying the total number of amino acids in SEQ ID NO:3 or 4 by the integer defining the percent identity divided by 100 and then subtracting that product from said total number of amino acids in SEQ ID NO:3 or 4, or:

$$25 N_a = X_a - (X_a * y),$$

wherein  $N_a$  is the number of amino acid alterations,  $X_a$  is the total number of amino acids in SEQ ID NO:3 or 4,  $y$  is, for instance 0.70 for 70%, 0.80 for 80%, 0.85 for 85% etc., and \* is the symbol for the multiplication operator, and wherein any non-integer product of  $X_a$  and  $y$  is rounded down to the nearest integer prior to

30 subtracting it from  $X_a$ .

"Fusion protein" refers to a protein encoded by two, often unrelated, fused genes or fragments thereof. In one example, EP-A-0 464 discloses fusion

proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, employing an immunoglobulin Fc region as a part of a fusion protein is advantageous for use in therapy and diagnosis resulting in, for example, improved 5 pharmacokinetic properties [see, e.g., EP-A 0232 262]. On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified.

“Small Molecule” or “Small Molecule Compound” refers to a synthetic organic compound that is typically less than about 2 kilodaltons (KD).

10 The term "transgene" is used herein to describe genetic material that has been or is about to be artificially inserted into the genome of a mammalian cell, particularly a mammalian cell of a living animal. The transgene is used to transform a cell, meaning that a permanent or transient genetic change, preferably a permanent genetic change, is induced in a cell following incorporation of 15 exogenous DNA. A permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACs, and the like. Of interest are transgenic mammals, e.g. cows, pigs, goats, horses, etc., and particularly rodents, e.g. rats, mice, etc.

20 Transgenic animals include an exogenous nucleic acid sequence present as an extrachromosomal element or stably integrated in all or a portion of its cells, especially in germ cells. Unless otherwise indicated, it will be assumed that a transgenic animal includes stable changes to the germline sequence. During the initial construction of the animal, "chimeras" or "chimeric animals" are generated, 25 in which only a subset of cells have the altered genome. Chimeras are primarily used for breeding purposes in order to generate the desired transgenic animal. Animals having a heterozygous alteration are generated by breeding of chimeras. Male and female heterozygotes are typically bred to generate homozygous animals.

30 In the present invention, "knock-outs" have a partial or complete loss of function in one or both alleles of the endogenous gene of interest, as opposed to "knock-ins" which have an introduced transgene with altered genetic sequence and function from the endogenous gene. The two may be combined, such that the naturally occurring gene is disabled, and an altered form introduced. In a knock-

out, preferably the target gene expression is rendered undetectable or insignificant.

A knock-out of a TIRAP gene means that function of TIRAP has been substantially decreased so that expression is not detectable or only present at insignificant levels. This may be achieved by a variety of mechanisms, including introduction of a

5 disruption of the coding sequence, e.g. insertion of one or more stop codons, insertion of a DNA fragment, etc., deletion of coding sequence, substitution of stop codons for coding sequence, etc. In some cases the exogenous transgene sequences are ultimately deleted from the genome, leaving a net change to the native sequence. Different approaches may be used to achieve the knock-out. A  
10 chromosomal deletion of all or part of the native gene may be induced, including deletions of the non-coding regions, particularly the promoter region, 3' regulatory sequences, enhancers, or deletions of gene that activate expression of genes. A functional knock-out may also be achieved by the introduction of an anti-sense construct that blocks expression of the native genes (for example, see Li and Cohen  
15 (1996) Cell 85:319-329). Knock-outs also include conditional knock-outs, for example where alteration of the target gene occurs upon exposure of the animal to a substance that promotes target gene alteration, introduction of an enzyme that promotes recombination at the target gene site (e.g. Cre in the Cre-lox system), or other method for directing the target gene alteration postnatally.

20 All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

25 The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

### 30 EXAMPLES

Example 1. TIRAP is a component of the TLR4 signaling pathway, but not of the IL-1R signaling pathways.

To determine the effect of various TIRAP constructs on the promoter activity of NF- $\kappa$ B, 293T cells were co-transfected with 2.0  $\mu$ g of the indicated TIRAP construct and 0.1  $\mu$ g of an NF- $\kappa$ B-dependent luciferase reporter gene. pcDNA3 is an empty vector control; TIRAP is a vector encoding the full length TIRAP protein; TIRAP N is a vector encoding the N-terminal domain of TIRAP; TIRAP C is a vector encoding the C terminal domain of TIRAP; and TIRAP P125H is a vector encoding a mutant form of TIRAP containing a proline to histidine mutation at amino acid position 125. Cells were harvested 24h post-transfection and lysed for analysis in a standard luciferase reporter assay. Luciferase activity is expressed as fold induction relative to mock transfected cells (Figure 2A). Whereas the TIRAP construct resulted in strong activation of the NF- $\kappa$ B promoter (approximately 80-fold induction of luciferase activity); no significant promoter activity was observed in the presence of TIRAP N, TIRAP C, or TIRAP P125H. Based on these results, full length TIRAP, but not the mutant forms of TIRAP tested, activates the NF- $\kappa$ B promoter. These results further suggest that TIRAP is a component of the TLR4 signaling pathway.

To determine whether TIRAP P125H functions as a dominant negative, 293T cells were transfected with 0.25  $\mu$ g of vector alone (pcDNA3) or CD4/TLR4 (a vector expressing a constitutively active form of TLR4) with or without increasing concentrations (50 ng, 250 ng, 1.0  $\mu$ g) of either TIRAP P125H or MyD88 DN (a vector expressing MyD88 containing a proline to histidine mutation at the same position as TIRAP P125H) and the NF- $\kappa$ B-dependent luciferase reporter gene. Interestingly, TIRAP P125H strongly inhibited activation of NF- $\kappa$ B mediated by CD4/TLR4 (Figure 2B). This inhibition was dose-dependent and comparable to the inhibition of CD4/TLR4 by MyD88 DN. These results suggest that TIRAP P125H inhibits TLR4-mediated NF- $\kappa$ B activation. Notably, a MyD88 mutation at the same position can still activate NF- $\kappa$ B when overexpressed, indicating that there may be differences in the function of the TIRAP and MyD88 TIR domains.

Unlike TLR4, neither IL-1R nor TLR9 can induce NF- $\kappa$ B in the absence of MyD88 (Kawai 1999; Schnare 2000; Adache 1998). To assess the possibility that TIRAP is the putative adapter in the MyD88-independent signaling

pathway, we tested TIRAP P125H for its ability to inhibit NF- $\kappa$ B activity induced by IL-1R and TLR9. 293T cells were co-transfected with either vector alone (pcDNA3), IL-1R (0.1  $\mu$ g), and IL-1R AcP (0.1  $\mu$ g) [Dr. Medzhitov: Please indicate what the IL-1R AcP construct encodes] plasmid cDNAs, with or without 5 increasing concentrations (10 ng, 50 ng, 200 ng) of TIRAP P125H plasmid and the NF- $\kappa$ B-dependent luciferase reporter gene. TIRAP P125H did not inhibit NF- $\kappa$ B activity induced either by co-transfected IL-1R and IL-1R AcP (Figure 2C) or by IL-1B cytokine.

TLR9 does not induce NF- $\kappa$ B activity when overexpressed, but does 10 so upon addition of its ligand CpG DNA. To determine whether TIRAP inhibits NF- $\kappa$ B activation induced by CpG-stimulation of TLR9-transfected cells, 293T cells were transfected with vector alone (pcDNA3), TLR9 (50 ng), with or without increasing concentrations (100 ng, 250 ng) of either TIRAP P125H or MyD88 DN. At twenty-four h post transfection, CpG oligonucleotides were added at 3  $\mu$ M final 15 concentration to TLR9-transfected samples and incubated for 14 more hours before harvesting. Whereas MyD88 DN inhibited CpG signaling, consistent with the ability of CpG DNA to activate NF- $\kappa$ B in MyD88-deficient cells (Schnare 2000), TIRAP P125H did not inhibit NF- $\kappa$ B activation induced by CpG-stimulation of TLR9-transfected cells (Figure 2D). Thus, TIRAP appears to function downstream 20 of TLR4 but not IL-1R or TLR9, and is likely to be the adapter in the MyD88-independent pathway downstream of TLR4.

Example 2. Wild-type TIRAP but not TIRAP P125H co-immunoprecipitates with TLR4.

To obtain biochemical conformation for a role of TIRAP in the 25 TLR4 signaling pathway, TIRAP-TLR4 protein interactions were evaluated using co-immunoprecipitation experiments. 293T cells were co-transfected with 4.5  $\mu$ g of a Myc-tagged TLR4 deletion construct lacking the ectodomain (Myc TLR4 wt) or its mutant derivative (Myc TLR4 P714H) in the presence or absence of 1.5  $\mu$ g of Flag-tagged TIRAP (Flag TIRAP) or its mutant derivative containing the P125H 30 mutation (Flag TIRAP PH). Lysates prepared 24h post-transfection were incubated overnight with anti-Flag M2 agarose beads to immunoprecipitate Flag TIRAP or Flag TIRAP P125H and any associated proteins. Immunocomplexes were washed,

separated by SDS-PAGE electrophoresis, and then analyzed by immunoblotting with either anti-myc antibody to look for co-immunoprecipitated TLR4 or anti-Flag antibody to confirm immunoprecipitation of Flag TIRAP or Flag TIRAP P125H. Total cell lysates (35 µg/sample) were likewise analyzed by SDS-PAGE and immunoblotting to confirm equivalent expression of the appropriate proteins in every sample. These experiments revealed that TLR4 does in fact co-immunoprecipitate with TIRAP (Figure 3A). The TLR4 mutant, TLR4 P714H, which cannot signal as a result of the mutation and thus, renders c3H/HeJ mice unresponsive to LPS, also co-immunoprecipitates with TLR4. In contrast, TIRAP P125H failed to co-immunoprecipitate with TLR4, suggesting that this conserved region of the TIR domain mediates interaction of TIRAP with the TIR domain of TLR4 (Figure 3A).

Similarly, the interaction between TIRAP and MyD88 was examined. 293T cells were transfected with 1.5 µg of either HA-tagged TIRAP (HA TIRAP) alone or in combination with 1.5 µg of either Flag-tagged MyD88 (Flag MyD88), Flag-tagged TIRAP (Flag-TIRAP), or Flag-tagged TIRAP P125H (Flag TIRAP PH). Immunoprecipitation and analyses of immunocomplexes as well as total cell lysates were done as described above. It was demonstrated that TIRAP does not interact with MyD88 (Figure 3B), demonstrating the specificity of the TIR domain interactions detected by these assays. TIRAP also failed to associate with IRAK [Dr. Medzhitov: Please spell this out], suggesting that TIRAP may not have any role in the MyD88-dependent signaling pathway. However, TIRAP can be co-immunoprecipitated with itself (Figure 3B), indicating that TIRAP can homodimerize, as is the case with many other TIR domain-containing proteins including TLR4 and MyD88. Direct interactions between TIRAP and the TIR domain of TLR4 was also detected using in vitro glutathione S transferase (GST) pull-down assays.

Example 3. PKR is a component of LPS and CpG-signaling pathways, and co-immunoprecipitates with TIRAP.

It was recently demonstrated that cells lacking the interferon-regulated, dsRNA-binding protein kinase, PKR, fail to activate JNK and p38 in response to LPS stimulation (Goh 2000). As TLR4 is required for all signals downstream of LPS, this results report suggested that PKR is a component of the

TLR4 signaling pathway. To determine whether PKR functions in the MyD88-dependent or -independent pathway, PKR activation was evaluated in wild-type and MyD88-deficient macrophages following LPS stimulation. To this end, bone marrow-derived macrophages from either wild-type or MyD88-deficient mice were 5 stimulated for the indicated time periods with either LPS (10 ng/ml) or CpG (10  $\mu$ M), then washed in PBS and lysed in TNT lysis buffer. Because PKR is phosphorylated on a threonine residue upon activation, activation of PKR was assayed by immunoblotting lysates (40  $\mu$ g/sample), resolved by SDS-PAGE electrophoresis, with an antibody that specifically recognizes phosphorylated PKR. 10 Activated, phosphorylated PKR was detectable in LPS-stimulated wild-type macrophages (Figure 4A). Interestingly, phosphorylated PKR was also detected in LPS-stimulated MyD88-deficient macrophages, although a significantly smaller fraction of PKR is phosphorylated in these cells. In addition, MyD88-deficient cells activate PKR with slower kinetics, similar to the delayed kinetics of NF- $\kappa$ B 15 and JNK activation. These observations strongly suggest that, in response to LPS stimulation, PKR is activated by MyD88-dependent as well as MyD88-independent mechanisms. If the MyD88-dependent pathway can activate PKR in response to LPS, it is conceivable that PKR may be activated by other TLRs. Therefore, PKR activation in response to CpG stimulation was tested. Indeed, PKR is 20 phosphorylated in response to CpG (Figure 4A), indicating that PKR is a component of the TLR9 pathway as well as the TLR4 pathway.

To determine whether PKR and TIRAP interact, lysates from 293T cells transfected with either 3.5  $\mu$ g of an HA PKR kinase dead (KD) mutant or 1.5  $\mu$ g of Flag TIRAP were subjected to immunoprecipitation analyses as described 25 above, followed by SDS-PAGE and immunoblotting with the indicated antibodies. The PKR KD mutant rather than wild type PKR was used because overexpressing PKR induces cell death. PKR did, in fact, co-immunoprecipitate with TIRAP (Figure 4B), further supporting the link between PKR and TIRAP established above.

30 Because TIRAP may not regulate PKR directly, the interaction between TIRAP and two other proteins, p58 and PACT, was analyzed. p58 is a tetratricopeptide repeat-containing protein that negatively regulates PKR activity (Lee 1994), and PACT is a dsRNA-binding domain-containing protein that

activates PKR in response to cellular stress (Patel 19980). Lysates from 293T cells transfected with either p58-encoding vector (3.5  $\mu$ g) or PACT (3.5  $\mu$ g) and Flag TIRAP (1.5  $\mu$ g) were subject to immunoprecipitation analyses followed by SDS-PAGE and immunoblotting with the indicated antibodies. TIRAP was shown to 5 immunoprecipitate with both p58 and PACT (Figure 4C), indicating that PKR and/or its regulators p58 and PACT may be downstream targets of TIRAP.

Example 4. The cell permeable TIRAP inhibitory peptide inhibits LPS but not CpG induced NF- $\kappa$ B activation, PKR phosphorylation, and JNK phosphorylation.

To further elucidate the function of TIRAP, alternative methods of 10 TIRAP inhibition were sought. The fact that the TIRAP P125H mutant functioned as a dominant negative and could no longer interact with TLR4 indicated that the mutated residue is critical for TIRAP activity, including its ability to interact with the upstream receptor. Therefore, a peptide corresponding to this region of wild-type TIRAP may act as a site-specific inhibitor of TIRAP by competing with 15 TIRAP for interaction with TLR4. To test this possibility, a cell-permeable peptide, useful for cellular assays, was first constructed. A synthetic peptide corresponding to this region of mTIRAP was linked to the C-terminus of a peptide derived from antennapedia. The antennapedia peptide can cross cell membranes when added exogenously to cell cultures, and serves as a vehicle to deliver the fusion peptide 20 into the cell (Derossi 1998). A RAW macrophage cell line stably transfected with an NF- $\kappa$ B-dependent luciferase reporter gene (RAW  $\kappa$ B) was pretreated for 1 hour with the indicated concentrations of either the TIRAP inhibitory peptide or 50 $\mu$ M of control peptide, then stimulated for 5 hours with LPS (10 ng/ml) before harvesting for the reporter assay. Samples were treated and stimulated in 25 duplicates. Pretreatment of the RAW  $\kappa$ B cell line with the TIRAP peptide potently inhibited LPS-induced NF- $\kappa$ B activation (Figure 5A). However, no effect on NF- $\kappa$ B activation was observed upon pretreatment with either the solvent, DMSO, or a control peptide in which the TIRAP sequence was reversed (Figure 5A).

To determine the effect of TIRAP on I $\kappa$ B- $\alpha$  degradation, the 30 following experiment was performed. RAW cells, either untreated or pretreated for 1 hour with 40  $\mu$ M of the TIRAP peptide, were either left unstimulated or stimulated with LPS (10 ng/ml) for the indicated time periods before harvesting for

lysis in TNT lysis buffer. Lysates (30 µg/sample) were resolved by SDS-PAGE electrophoresis followed by immunoblotting with an anti-IκB-α degradation. Consistent with the block in NF-κB activation demonstrated above, LPS stimulation did not induce IκB-α degradation in RAW cells pretreated with the 5 TIRAP peptide (Figure 5B).

To determine whether a TIRAP mutant peptide inhibits CpG-stimulated signaling, RAW κB cells were pretreated with the indicated concentrations of the TIRAP peptide and stimulated for 5 hours with either LPS (10 ng/ml) or CpG (10 µM) before harvesting for reporter assay. Importantly, 10 pretreatment of RAW cells with the TIRAP peptide did not inhibit either CpG-induced NF-κB activation (Figure 5C) or CpG-induced IκB-α degradation, nor did the peptide inhibit NF-κB activation mediated by IL-1B cytokine in 293 cells.

To determine whether a TIRAP mutant peptide inhibits PKR and JNK phosphorylation induced by LPS or CpG, RAW cells either untreated or 15 pretreated with 40 µM of the TIRAP peptide were either left unstimulated or stimulated with either LPS (10 ng/ml) or CpG (10 µM) for the indicated time periods before harvest and lysis. Lysates (30 µg/sample) were analyzed by SDS-PAGE followed by immunoblotting with antibodies that specifically recognize phosphorylated PKR and JNK. Furthermore, the TIRAP peptide, but not the 20 control peptide, also inhibited PKR phosphorylation induced by LPS, but not CpG, in RAW cells (Figure 5D), supporting the possibility that PKR functions downstream of TIRAP.

These data are consistent with the results from transient transfection assays which utilized the TIRAP P125H dominant negative construct, and they 25 strongly support the hypothesis that TIRAP functions downstream of TLR4, but not TLR9 or IL-1R. Moreover, the fact that the TIRAP peptide does not inhibit CpG-induced effects rules out the possibility that the TIRAP peptide inhibits LPS signaling by causing cytotoxicity or by interfering non-specifically with other cellular processes.

30 Example 5. TIRAP controls dendritic cell maturation.

In view of the important role for TIRAP in mediating LPS-responsiveness in macrophages, the role of TIRAP in LPS-induced DC maturation was investigated. TIRAP is likely to be critical in these events because MyD88-deficient DCs retain the ability to upregulate costimulatory molecules in response to LPS stimulation (Kaisho 2001). To determine the effect of TIRAP on the expression of the costimulatory molecules B7.1 and B7.2, flow cytometry was performed on wild type and MyD88-/-DC. DCs were either unstimulated (shaded), stimulated with LPS (10 ng/ml) or CpG (10  $\mu$ M) (black line), or pretreated with TIRAP peptide (10  $\mu$ M) prior to stimulation with LPS or CpG (dashed line). Indeed, the TIRAP peptide abolished LPS-induced upregulation of B7.1 and B7.2 molecules in both wild-type and MyD88-deficient DCs (Figure 6A). However, the peptide had no effect on CpG-induced DC maturation (Figure 6A).

To determine the effects of the TIRAP peptide on cytokine production, DCs were treated with or without TIRAP peptide prior to stimulation with LPS or CpG (as described above), and the production of IL-12 and IL-6 was measured. Addition of TIRAP peptide inhibited production of IL-12 and IL-6 cytokines in response to stimulation with LPS, but not CpG (Figure 6B).

Lastly, the effect of TIRAP peptide on T cell proliferation was evaluated. Stimulation of CD4 T cell proliferation by DCs treated with or without TIRAP peptide prior to stimulation with LPS was performed in the presence of varying concentrations of E $\alpha$  peptide. DCs were fixed prior to incubation with 1H3.1 TCR transgenic T cells (specific for E $\alpha$  peptide bound to I-A). Proliferation was measured by  $^3$ H-thymidine incorporation for the last 24h of a 48h culture. TIRAP peptide-treated DCs from either wild-type or MyD88-deficient mice were unable to induce T cell proliferation (Figure 6C). These data demonstrate that TIRAP can activate a pathway that induces DC maturation and suggest that TIRAP is responsible for the LPS-induced maturation in MyD88-deficient DCs. As these results indicate, TIRAP appears to be critical for the ability of DCs to produce cytokines, upregulate costimulatory molecules, and prime naïve T cells in response to LPS stimulation.

#### Example 6. Generation of TIRAP Knock-Out Mice

To make the targeting construct, 7.4 kb of the mouse TIRAP gene was cloned by RT-PCR and subcloned into a pEasy Flox vector. The second exon of the mouse TIRAP gene, which encodes 610 bp of the 730 bp-coding sequence, was then replaced with neomycin in the pEasy Flox vector using techniques standard in the art. It is our current understanding that the 3.7 kb of the 5' flanking sequences and 3.1 kb of the 3' flanking sequences direct homologous recombination at the mouse TIRAP locus.

10 Embryonic stem cells were then transfected with the targeting construct by electroporation, and selected using G418 and gangcyclovir. To identify clones that have integrated the targeting construct by homologous recombination, clones were screened by Southern analysis. Two positive clones were then chosen for injection into pseudopregnant mothers.

15 From these injected clones developed two highly chimeric male mice, which were bred with female littermates. Tail DNAs were then obtained from pups for Southern analysis. Germline transmission was obtained with two mice. Lastly, these two F1 mice were interbred to generate homozygous TIRAP knockout mice, which were born at the expected Mendelian frequency.

Example 7. Exposure of Cells of TIRAP Knock-Out Mice to TLR Ligands

20 Figure 8 graphically represents deficient B cell proliferation in cells from the TIRAP knock-out mice produced in the manner described in Example 6. Splenocytes were taken from wild-type and the TIRAP knock-out mice, counted and plated into 96 well plates at about 100,000 cells/100 ml. Serial dilutions of LPS (starting from 20 ug./ml. final concentration) and BLP (starting from 300 ng./ml. final concentration) were added to the splenocytes. Thirty-six hours later, 25 radioactive  $^3$ H thymidine was added to each well. The incorporation of  $^3$ H thymidine into proliferating B cells was assessed 12 hours later by use of a beta counter. As the results shown Figure 8 indicate, the amount of  $^3$ H thymidine incorporated into the cells, and thus the B cells' proliferation, was significantly reduced in the TIRAP knock-out mice splenocytes as compared to the wild-type 30 mouse splenocytes.

Figure 9 graphically represents cytokine production by dendritic cells from TIRAP knock-out mice produced in the manner described in Example 6.

Bone marrow-derived cells were taken from the wild-type and TIRAP knock-out mice, and were cultured for five days in complete RPMI supplemented with GM-CSF in 24-well plates. On day 5, the cells were counted and replated into 24-well plates at about 100,000 cells/ml., and stimulated overnight with LPS (10 ng./ml.) or 5 BLP (100 ng./ml.). Twenty-four hours post stimulation, supernatants were harvested for ELISA.

For the ELISA analysis, plates were coated with appropriate capture antibodies, namely IL-6 or TNF- $\alpha$ , washed, and then coated with supernatants from the various samples. After overnight incubation, the supernatant was washed off, 10 and the appropriate biotin-conjugated detection antibody was added. Streptavidin-HRP was then added, followed by its substrate. Colorimetric changes were monitored using an ELISA plate reader. Quantification of cytokine concentrations in supernatants was done by comparison with a standard made using the respective recombinant cytokines. As the results shown Figure 9 indicate, the amount of 15 cytokine production by dendritic cells was significantly reduced in the TIRAP knock-out mice dendritic cells as compared to the wild-type mouse dendritic cells.

What is claimed is:

1. An isolated polynucleotide comprising the nucleotide sequence of SEQ ID NO:1.
2. An expression vector comprising a polynucleotide that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:3 when said expression vector is present in a compatible host cell.
- 5 3. A process for producing a recombinant host cell comprising the step of introducing said expression vector of claim 2 into a host cell such that said host cell, under appropriate culture conditions, produces said polypeptide.
- 10 4. A recombinant host cell produced by the process of claim 3.
5. A membrane of a recombinant host cell of claim 4, wherein said cell expresses said polypeptide.
6. A process for producing a polypeptide comprising culturing a recombinant host cell of claim 4 under conditions sufficient for the production of said polypeptide
- 15 and recovering said polypeptide from the culture.
7. An isolated polynucleotide comprising a polynucleotide sequence that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:3.
8. An isolated polynucleotide comprising a polynucleotide sequence that is fully complementary to a nucleotide sequence that encodes a polypeptide comprising the
- 20 amino acid sequence of SEQ ID NO:3.
9. An isolated polynucleotide comprising a polynucleotide sequence that is fully complementary to the nucleotide sequence of SEQ ID NO:1.
10. An isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:3.
- 25 11. The polypeptide of claim 10 consisting of the amino acid sequence of SEQ ID NO:3.
12. An antibody immunospecific to the polypeptide of claim 10.
13. A polypeptide obtainable by expressing a nucleotide sequence comprising the sequence of SEQ ID NO:1.
- 30 14. An isolated polynucleotide comprising the nucleotide sequence of SEQ ID NO:5.

15. An isolated polynucleotide comprising a polynucleotide sequence that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:9.
16. An isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:9.
- 5 17. An isolated polynucleotide comprising the nucleotide sequence of SEQ ID NO:2.
18. An expression vector comprising a polynucleotide that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:4 when said expression vector is present in a compatible host cell.
- 10 19. A process for producing a recombinant host cell comprising the step of introducing said expression vector of claim 18 into a host cell such that said host cell, under appropriate culture conditions, produces said polypeptide.
20. A recombinant host cell produced by the process of claim 19.
21. A membrane of a recombinant host cell of claim 20, wherein said cell expresses said polypeptide.
- 15 22. A process for producing a polypeptide comprising culturing a recombinant host cell of claim 20 under conditions sufficient for the production of said polypeptide and recovering said polypeptide from the culture.
23. An isolated polynucleotide comprising a polynucleotide sequence that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:4.
- 20 24. An isolated polynucleotide comprising a polynucleotide sequence that is fully complementary to a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:4.
25. An isolated polynucleotide comprising a polynucleotide sequence that is fully complementary to the nucleotide sequence of SEQ ID NO:2.
26. An isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:4.
27. The polypeptide of claim 26 consisting of the amino acid sequence of SEQ ID NO:4.
- 30 28. An antibody immunospecific to the polypeptide of claim 26.
29. A polypeptide obtainable by expressing a nucleotide sequence comprising the sequence of SEQ ID NO:2.

30. An isolated polynucleotide comprising the nucleotide sequence of SEQ ID NO:5.
31. An isolated polynucleotide comprising a polynucleotide sequence that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:9.
- 5 32. An isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:9.
33. An isolated polynucleotide comprising a polynucleotide sequence that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:11.
34. An isolated polypeptide comprising an amino acid sequence set forth in SEQ 10 ID NO:11.
35. A drug screening method comprising the steps of
  - a. Providing a polypeptide comprising at least the TIRAP-binding domain of TLR4;
  - b. Providing a TIRAP polypeptide or other polypeptide that comprises a 15 TLR4 binding domain;
  - c. Providing a candidate compound;
  - d. Placing the candidate compound into proximity with the polypeptide comprising the TIRAP-binding domain of TLR4 and the TIRAP polypeptide or other polypeptide; and
  - 20 e. Determining whether the candidate compound competes with the binding of either of the polypeptide comprising the TIRAP-binding domain of TLR4 or the TIRAP polypeptide or other polypeptide.
36. The drug screening method of claim 35, wherein the TIRAP-binding domain of TLR4 comprises TLR4 and the TIRAP polypeptide or other polypeptide 25 comprises the polypeptide set forth in SEQ ID NO:9.
37. The drug screening method of claim 35, wherein the TIRAP-binding domain of TLR4 comprises TLR4 and the TIRAP polypeptide or other polypeptide comprises the polypeptide set forth in SEQ ID NO:10.
38. A method for blocking TIRAP signaling in a cell, said method comprising 30 administering to said cell an inhibitory amount of a TIRAP inhibitor.

39. The method of claim 38, wherein said TIRAP inhibitor is a polypeptide.
40. The method of claim 38, wherein said TIRAP inhibitor is a peptide mimetic.
41. The method of claim 39, wherein said polypeptide comprises an amino acid sequence selected from the group of amino acid sequences set forth in SEQ ID 5 NOS: 9, 11 and 12.
42. A fusion protein comprising a TIRAP inhibitory polypeptide and a second polypeptide useful for delivery of said fusion protein to a cell.
43. The fusion protein of claim 42, wherein said second polypeptide comprises Antennapedia.
- 10 44. A method for screening for antagonists of TIRAP activity which comprises screening for peptides or small molecules that inhibit the MyD88-independent signaling of TLR4.
45. A method of screening for antagonists of TIRAP activity which comprises screening for peptides or small molecules that inhibit binding of TIRAP to TLR4.
- 15 46. A method for screening for agonists of TIRAP activity which comprises screening for peptides or small molecules that activate the MyD88-independent signaling of TLR4.
47. A method of screening for agonists of TIRAP activity which comprises screening for peptides or small molecules that bind to the TIRAP binding site of 20 TLR4.
48. The method of claim 44 which comprises contacting a candidate TIRAP antagonist with a TLR4 polypeptide or a TIRAP binding domain thereof and with TIRAP or a TLR4 binding domain thereof, sequentially in any order or simultaneously, and determining whether or not the candidate antagonist inhibits 25 the binding of TIRAP or a TLR4 binding domain thereof with the TLR4 polypeptide or TIRAP binding domain thereof.
49. A TIRAP antagonist which comprises TIRAP having at least one mutation in the TLR4 binding domain thereof such that the mutant binds to TLR4 but does not induce MyD88-independent signaling of TLR4.

50. A TIRAP antagonist which comprises TIRAP having at least one mutation in the TLR4 binding domain thereof such that the mutant binds to TIRAP and prevents TIRAP binding to TLR4 but does not induce MyD88-independent signaling of TLR4.

5 51. The TIRAP antagonist of claim 49 which comprises at least one mutation in the region of amino acid 118 to 131 as measured in human TIRAP.

52. The TIRAP antagonist of claim 51 wherein the binding domain of TIRAP has a proline to histidine mutation at position 125.

53. A method of treating a disease state associated with TIRAP expression 10 which comprises internally administering a polypeptide having a mutation in the TLR4 binding domain thereof such that the polypeptide binds to TLR4 but does not induce MyD88-independent signaling of TLR4.

54. A TIRAP antagonist which is a small molecule that inhibits binding of TIRAP to TLR4.

15 55. A method of modulating the immune response in an animal which comprises internally administering a TIRAP antagonist.

56. A method of modulating the immune response in an animal which comprises internally administering a polynucleotide which is anti-sense to a polynucleotide encoding TIRAP.

20 57. A transgenic knock-out non-human comprising disruption in the endogenous toll/interleukin-1 receptor adapter protein gene, wherein said disruption has been introduced into its genome by homologous recombination with a DNA targeting construct in an embryonic stem cell such that the targeting construct is stably integrated in the genome of said non-human.

25 58. The transgenic knock-out non-human of claim 57, wherein said non-human is a mouse.

1/14

h TIRAP	1	W A S S T S L R A P G S R P K K P L G K M A D W F R Q T L E K K P K K R N S P	40
m TIRAP	1	M A S S S V R A S S T P S K K P R D K I A D W F R Q A L E K K P K K R M P I S Q	40
h TIRAP	41	E S T S S D A S Q P T S Q D - S E - - - L P P S L S S V T S - - - - -	66
m TIRAP	41	E S H L Y D G S Q T A T Q D G L S P S S C S S P E S R S S P S S C S	80
h TIRAP	67	- - - E S L P P T H A S D S G S S - R W S K D Y D V C V C H S E E D L V A A	100
m TIRAP	81	S G M S R T S R P T H V D S S S G R W S K D Y D V C V G H S E E D L E A A	120
h TIRAP	101	Q D L V S Y L E G S T A S T R C F L Q L R D A T P G G A V S E L C Q A L S S	140
m TIRAP	121	Q E E V S Y L E G S Q A S L R C F L Q L R D A A P G G A V S E L C Q A L S R S	160
h TIRAP	141	H C R V L E T P G F E Q D P W C K Y Q M L Q A L T E A P G A E G C T I P L L S	180
m TIRAP	161	H C R V L E T P G F E R D P W C K Y Q M L Q A L T E A P G A E G C T I P L L S	200
h TIRAP	181	G Y S R A A Y P P E L R F M X Y W D G R G P D G G F R Q V K E A V M R C K L L Q	220
m TIRAP	201	G L S R A A Y P P E L R F M Y Y D G R G K D G G F Y Q N K E A V I H Y L E T L	240
h TIRAP	221	E G E G E R D S A T V S D L L	221
m TIRAP	241	S	241

FIG. 1

2/14

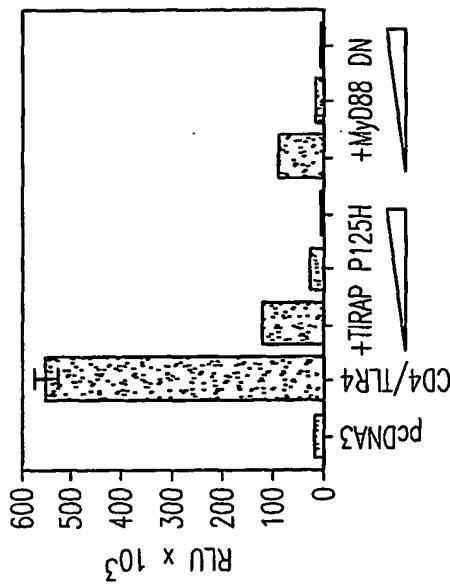


FIG. 2B

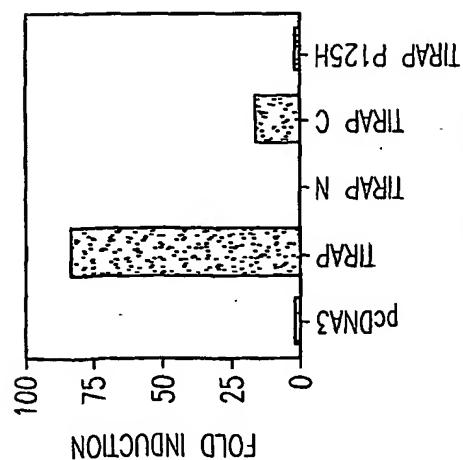


FIG. 2A

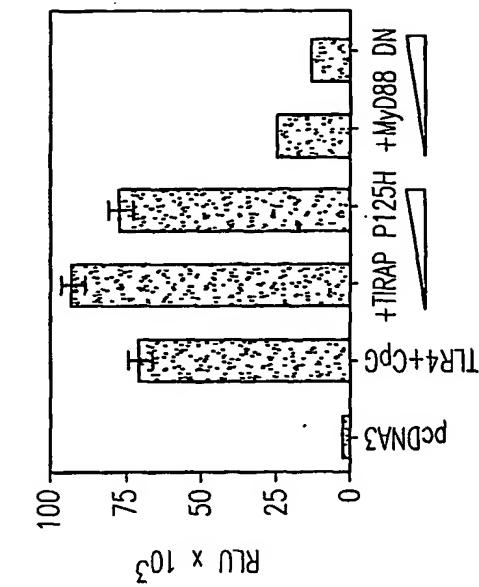


FIG. 2D

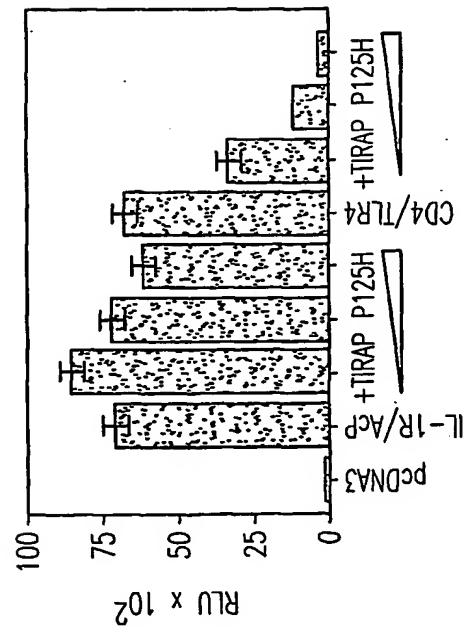


FIG. 2C

3/14

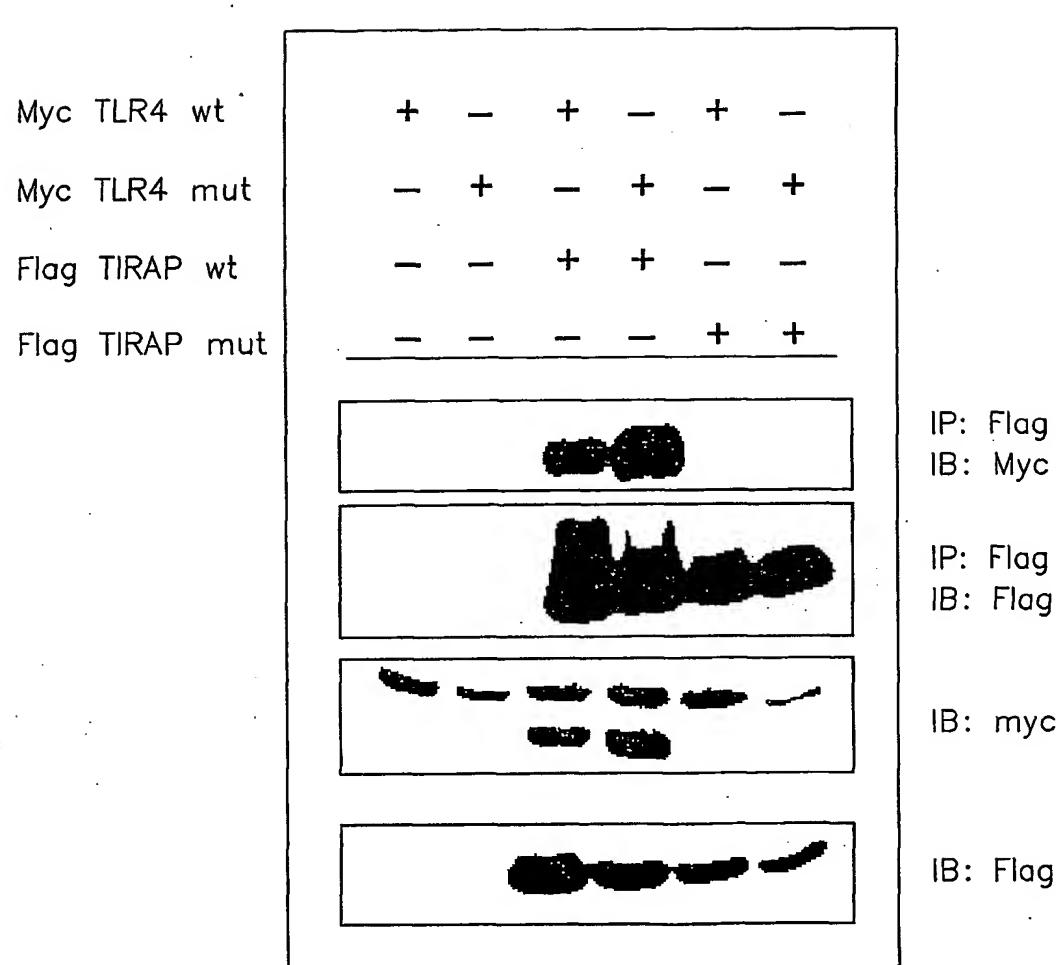


FIG. 3A

4/14

HA TIRAP	-	+	+	+	+
Flag MyD88	-	-	+	-	-
Flag TIRAP	-	-	-	+	-
Flag TIRAP P125H	-	-	-	-	+
	<hr/>				
IP: Flag					
IB: HA					
IB: HA					
IB: Flag					

FIG. 3B

5/14

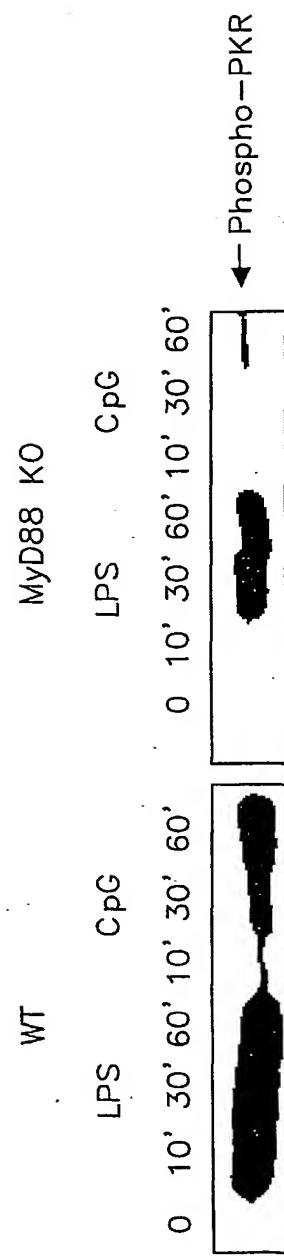


FIG. 4A

6/14

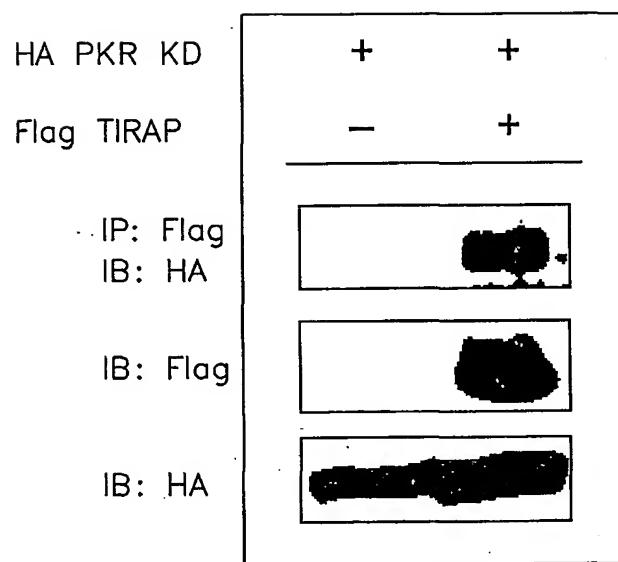


FIG. 4B

7/14

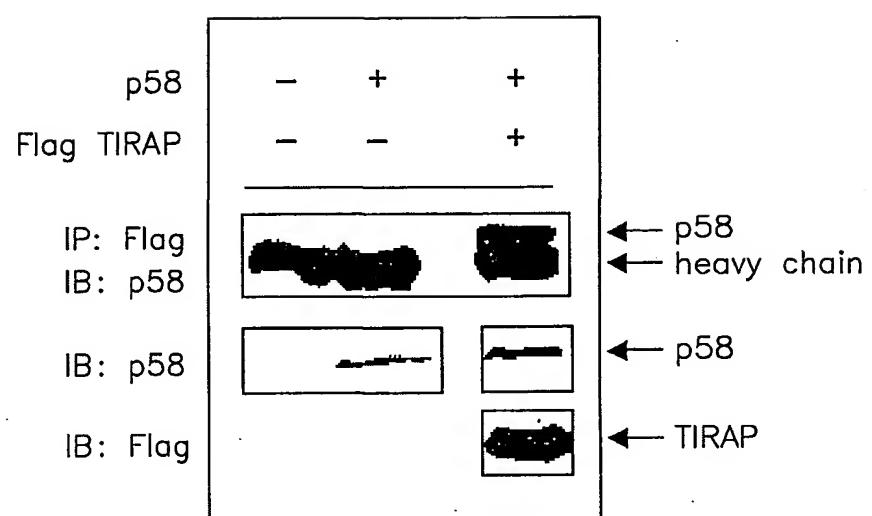


FIG. 4C

8/14

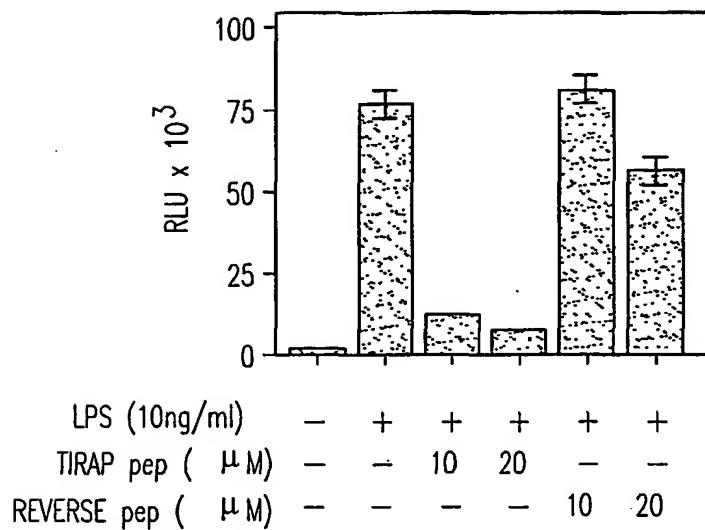


FIG. 5A

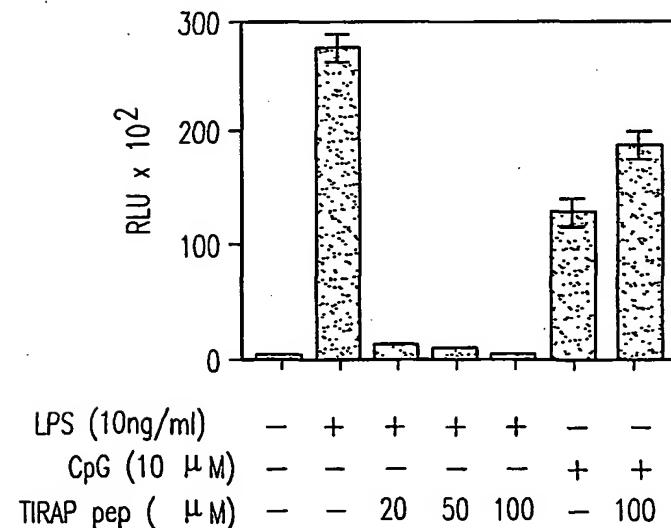


FIG. 5C

9/14

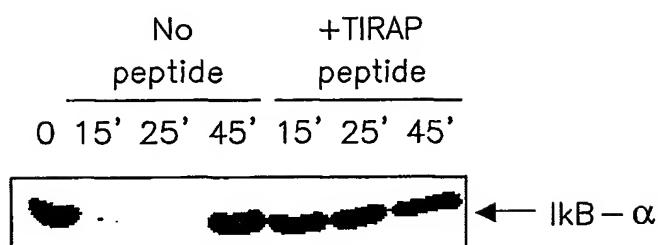


FIG. 5B

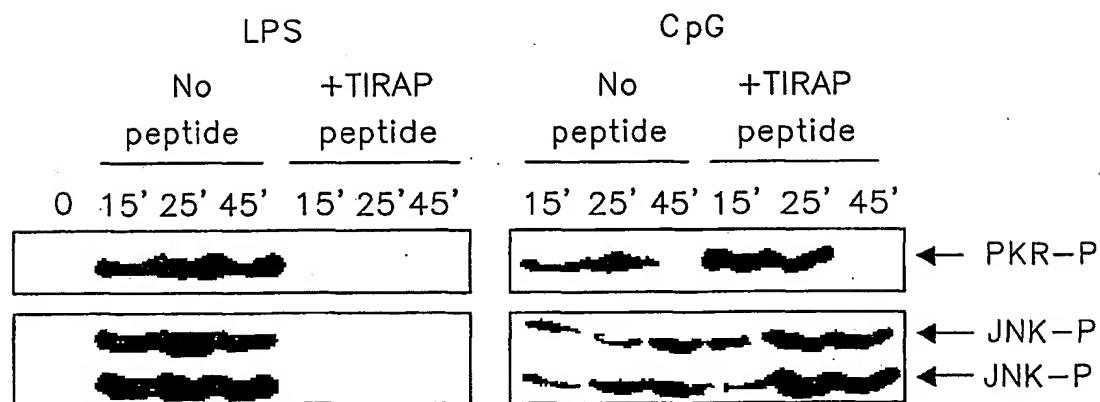


FIG. 5D

10/14

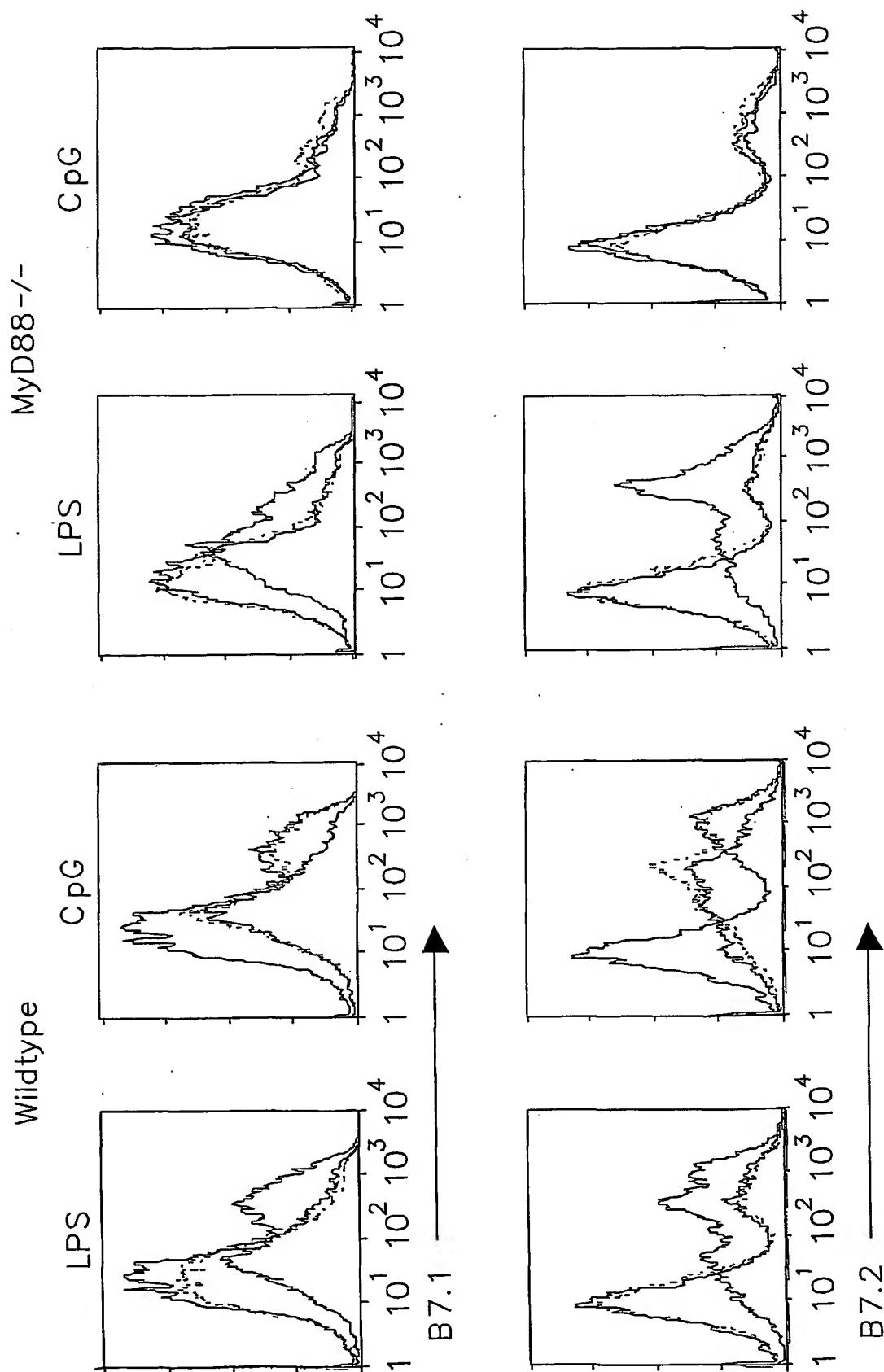


FIG. 6A

11/14

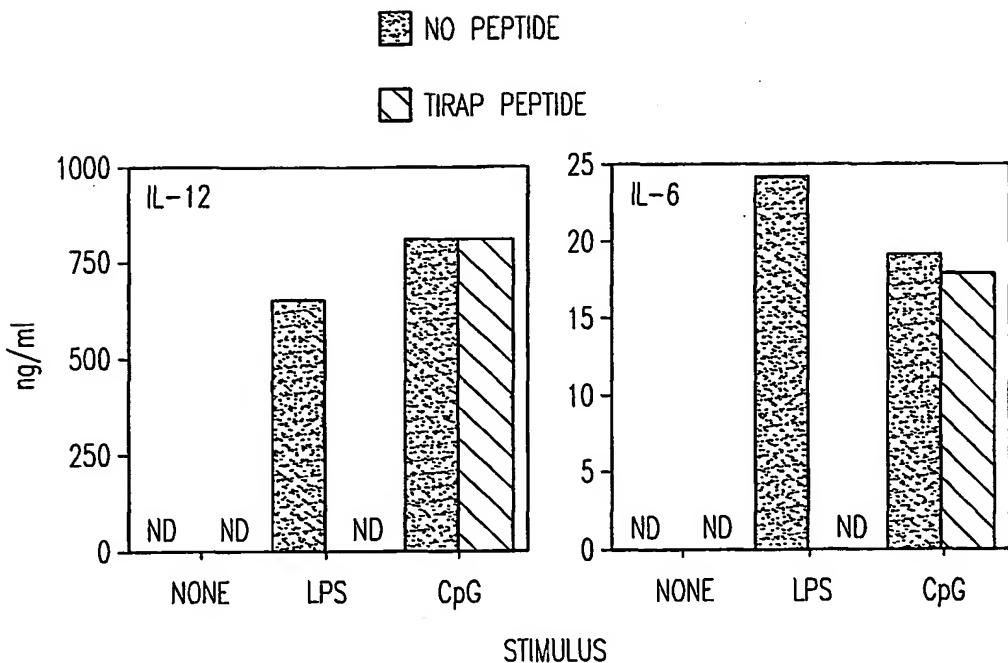
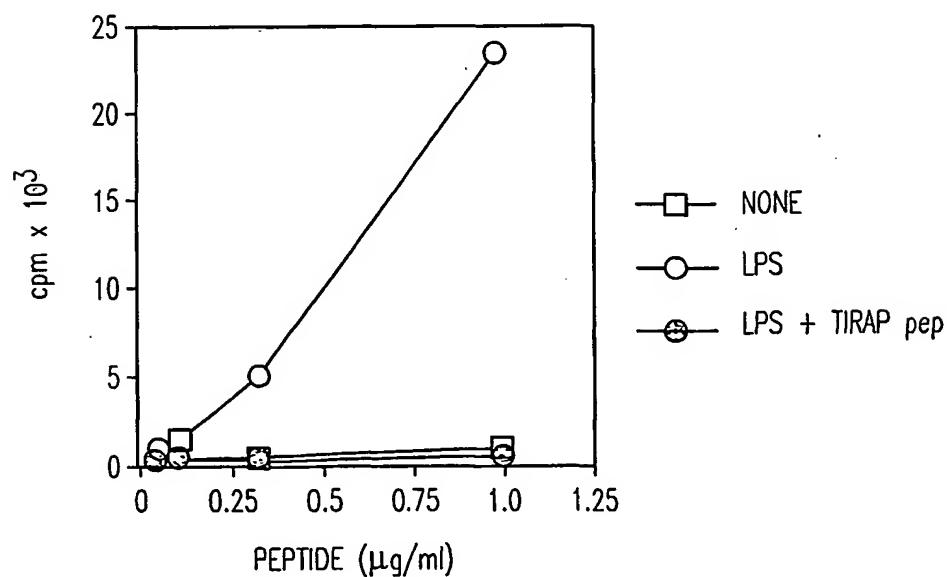


FIG. 6B



12/14

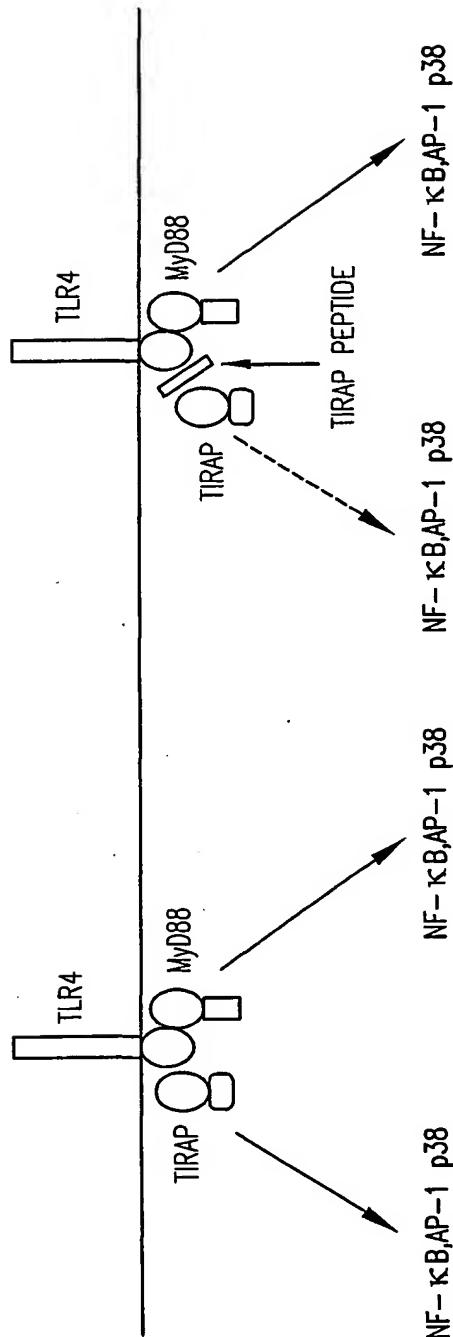


FIG. 7

13/14

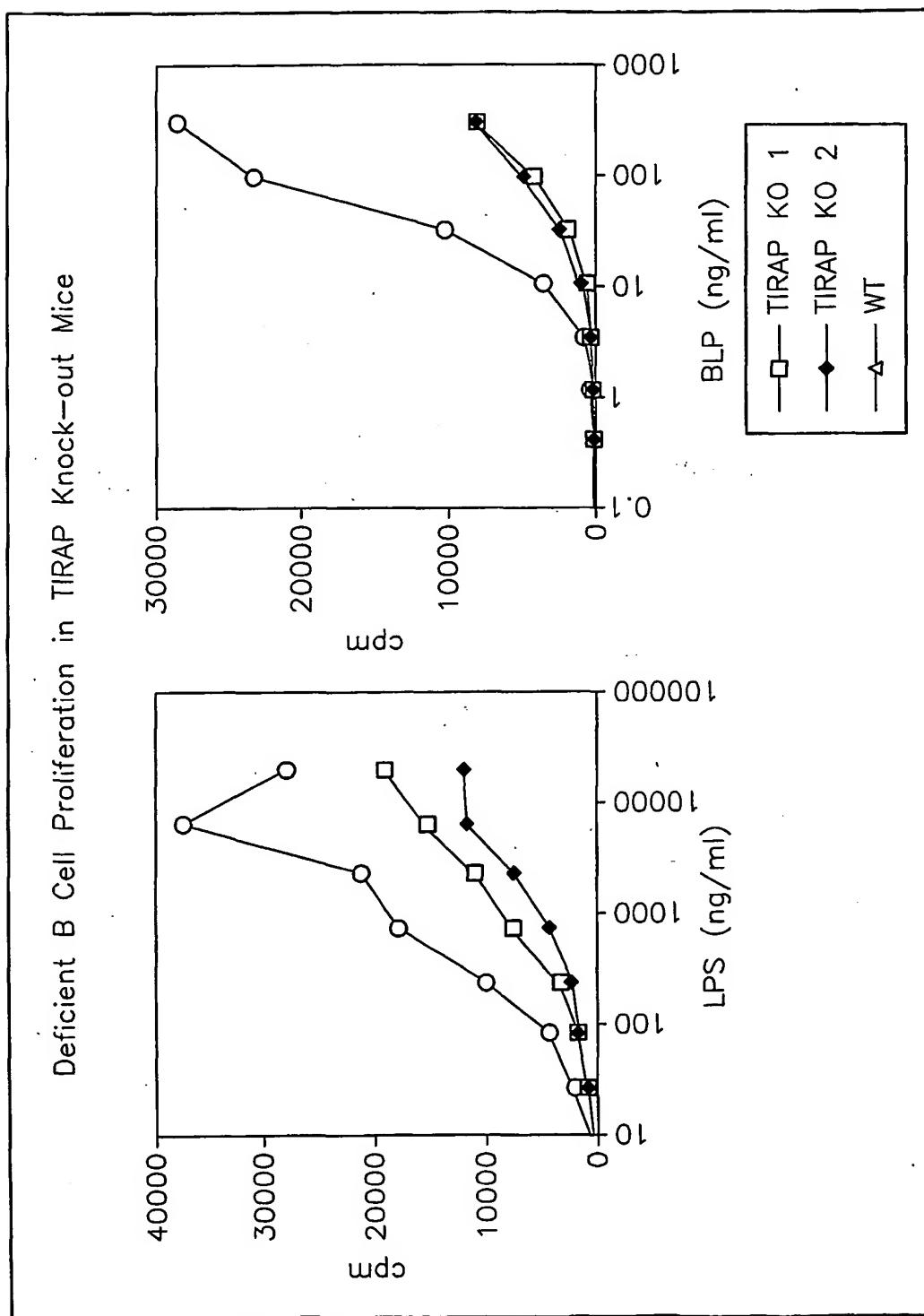


FIG. 8

14/14

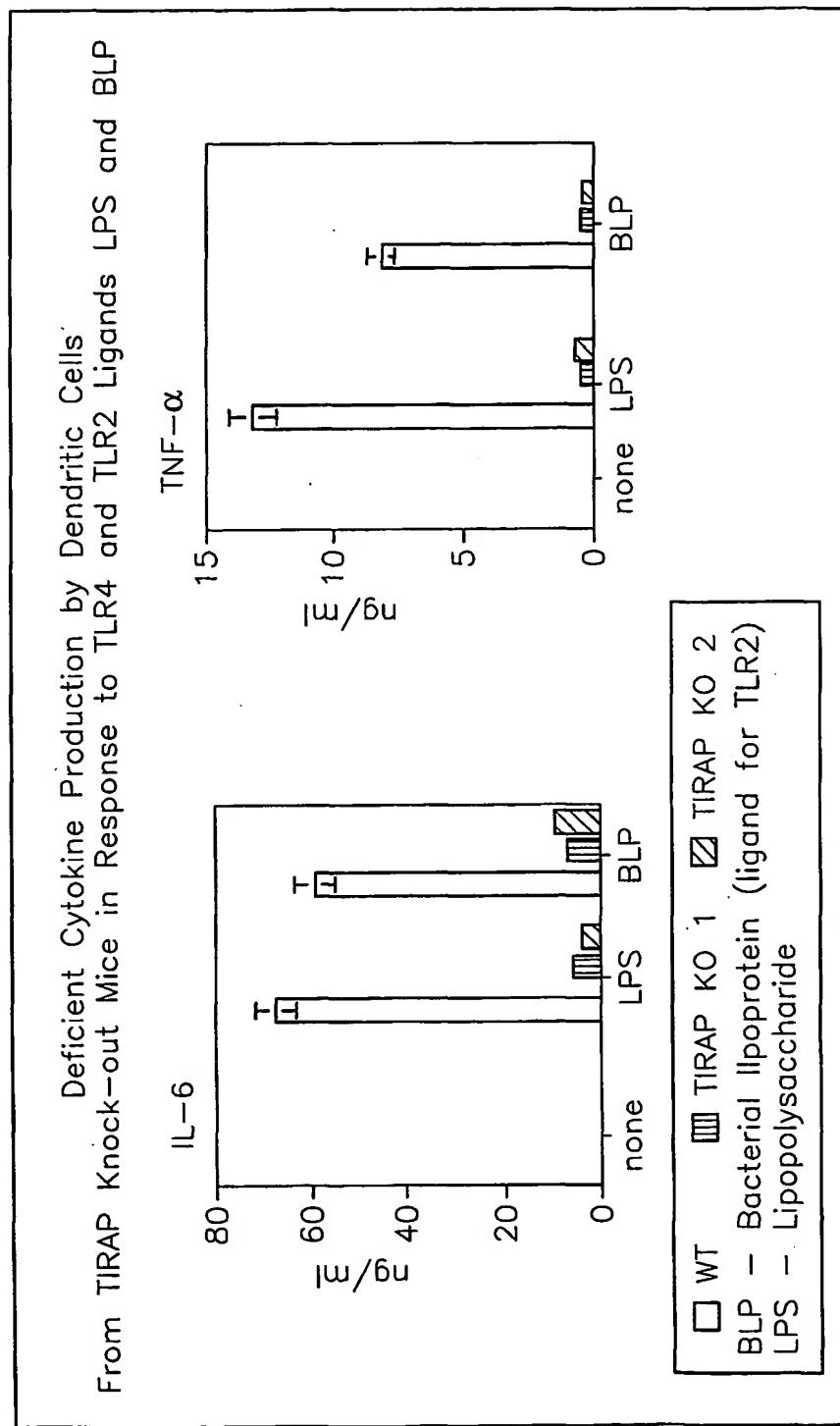


FIG. 9

## SEQUENCE LISTING

<110> YALE UNIVERSITY  
MEDHITOV, Ruslan  
HORNG, Tiffany  
BARTON, Gregory

<120> TOLL/INTERLEUKIN-1 RECEPTOR ADAPTER PROTEIN (TIRAP)

<130> 044574-5101WO

<150> 60/289,738  
<151> 2001-05-09

<150> 60/289,815  
<151> 2001-05-09

<150> 60/289,866  
<151> 2001-05-14

<160> 12

<170> PatentIn version 3.1

<210> 1  
<211> 708  
<212> DNA  
<213> Homo sapiens

<220>

<221> misc\_feature  
<223> TIRAP polynucleotide

<400> 1

atggcatcat cgacctccct cccagctcct ggctctcgcc ctaagaagcc tctaggcaag 60  
atggctgact gtttcaggca gacctcgctg aagaagccca agaagaggcc caactcccc 120  
gaaagcacct ccagcgatgc ttcacagcct acctcacagg acagcccaact acccccaagc 180  
ctcagctcag tcacgtctcc cagcctgcca cccacacatg cgagtgacag tggcagtagt 240  
cgctggagca aagactatga cgtctcgctg tgccacagtg aggaagacct ggtggccgccc 300  
caggacctgg ttccttactt ggaaggcagc actgccagcc tgcgctgctt cctgcaactc 360  
cgggatgcaa ccccaggcgg cgctatagtg tccgagctgt gccaggcaact gagcagtagt 420  
caactgccggg tgctgctcat cacgccgggc ttcccttcagg acccctggtg caagtaccag 480  
atgctgcagg ccctgaccga ggctccagggg gcccggggct gcaccatccc cctgctgtcg 540  
ggcctcagca gagctgccta cccacctgag ctccgattca tgtactacgt cgatggcagg 600  
ggccctgtatg gtggctttcg tcaagtcaaa gaagctgtca tgcgttgtaa gctactacag 660  
gagggagaag gggAACGGGA ttcaagctaca gtatctgtac tactttga 708

<210> 2  
<211> 726  
<212> DNA  
<213> Mus sp.

<220>

<221> misc\_feature  
<223> TIRAP polynucleotide

<220>  
 <221> misc\_feature  
 <222> (417)..(417)  
 <223> n=A,C,T,G

<400> 2  
 atggcttcat ctcctccgt cccagcctcc tccactccgt ccaagaagcc tcgagacaag 60  
 atagctgact gttcaggca ggctctgtt aagaagccca agaagatgcc gatctccag 120  
 gaaagccacc tctatgtatgg ttcacagaca gccacacagg atggctctc accctcgac 180  
 tgcagctcac ccccgagtca cagttcaccc gagagccgta gctaccctc gagctgcagt 240  
 tcaggaatgt cacctaccc gccaccaaca cacgtggaca gcagcagcag cagcagttgc 300  
 cgctggagca aagactacga tgtctgcgtg tgccacagtg aggaggactt ggaggcggcc 360  
 caggagctgg tctctactt ggagggtagc caggccagtc tacgctgctt cctgcanctt 420  
 cgggatgcag ccccggtgg cgccattgtt tcggagctat gccaggcact gagtcgttagt 480  
 cactgccgtg tgctgctcat cactccaggc ttccctcggg acccctggtg caagtaccag 540  
 atgctgcagg ccctgacgga ggcccccggcg tcggagggtt gcaccatacc cctgctgtcc 600  
 ggcctgtcca gagccgccta cccggcggaa ctccgattca tgtactatgt ggatggcaga 660  
 ggcaaggacg gaggcttttccaagtcaag gaggctgtt tacactatct ggagacacta 720  
 agctga 726

<210> 3  
 <211> 235  
 <212> PRT  
 <213> Homo sapiens

<220>  
 <221> MISC\_FEATURE  
 <223> TIRAP polypeptide

<400> 3  
 Met Ala Ser Ser Thr Ser Leu Pro Ala Pro Gly Ser Arg Pro Lys Lys  
 1 5 10 15

Pro Leu Gly Lys Met Ala Asp Trp Phe Arg Gln Thr Leu Leu Lys Lys  
 20 25 30

Pro Lys Lys Arg Pro Asn Ser Pro Glu Ser Thr Ser Ser Asp Ala Ser  
 35 40 45

Gln Pro Thr Ser Gln Asp Ser Pro Leu Pro Pro Ser Leu Ser Ser Val  
 50 55 60

Thr Ser Pro Ser Leu Pro Pro Thr His Ala Ser Asp Ser Gly Ser Ser  
 65 70 75 80

Arg Trp Ser Lys Asp Tyr Asp Val Cys Val Cys His Ser Glu Glu Asp  
 85 90 95

Leu Val Ala Ala Gln Asp Leu Val Ser Tyr Leu Glu Gly Ser Thr Ala  
 100 105 110

Ser Leu Arg Cys Phe Leu Gln Leu Arg Asp Ala Thr Pro Gly Gly Ala  
 115 120 125

Ile Val Ser Glu Leu Cys Gln Ala Leu Ser Ser Ser His Cys Arg Val  
 130 135 140

Leu Leu Ile Thr Pro Gly Phe Leu Gln Asp Pro Trp Cys Lys Tyr Gln  
 145 150 155 160

Met Leu Gln Ala Leu Thr Glu Ala Pro Gly Ala Glu Gly Cys Thr Ile  
 165 170 175

Pro Leu Leu Ser Gly Leu Ser Arg Ala Ala Tyr Pro Pro Glu Leu Arg  
 180 185 190

Phe Met Tyr Tyr Val Asp Gly Arg Gly Pro Asp Gly Gly Phe Arg Gln  
 195 200 205

Val Lys Glu Ala Val Met Arg Cys Lys Leu Leu Gln Glu Gly Glu Gly  
 210 215 220

Glu Arg Asp Ser Ala Thr Val Ser Asp Leu Leu  
 225 230 235

<210> 4

<211> 241

<212> PRT

<213> Mus sp.

<220>

<221> MISC\_FEATURE

<223> TIRAP polypeptide

<400> 4

Met Ala Ser Ser Ser Val Pro Ala Ser Ser Thr Pro Ser Lys Lys  
 1 5 10 15

Pro Arg Asp Lys Ile Ala Asp Trp Phe Arg Gln Ala Leu Leu Lys Lys  
 20 25 30

Pro Lys Lys Met Pro Ile Ser Gln Glu Ser His Leu Tyr Asp Gly Ser  
 35 40 45

Gln Thr Ala Thr Gln Asp Gly Leu Ser Pro Ser Ser Cys Ser Ser Pro  
 50 55 60

Pro Ser His Ser Ser Pro Glu Ser Arg Ser Ser Pro Ser Ser Cys Ser  
 65 70 75 80

Ser Gly Met Ser Pro Thr Ser Pro Pro Thr His Val Asp Ser Ser Ser  
 85 90 95

Ser Ser Ser Gly Arg Trp Ser Lys Asp Tyr Asp Val Cys Val Cys His  
 100 105 110

Ser Glu Glu Asp Leu Glu Ala Ala Gln Glu Leu Val Ser Tyr Leu Glu  
 115 120 125

Gly Ser Gln Ala Ser Leu Arg Cys Phe Leu Gln Leu Arg Asp Ala Ala  
 130 135 140

Pro Gly Gly Ala Ile Val Ser Glu Leu Cys Gln Ala Leu Ser Arg Ser  
 145 150 155 160

His Cys Arg Val Leu Leu Ile Thr Pro Gly Phe Leu Arg Asp Pro Trp  
 165 170 175

Cys Lys Tyr Gln Met Leu Gln Ala Leu Thr Glu Ala Pro Ala Ser Glu  
 180 185 190

Gly Cys Thr Ile Pro Leu Leu Ser Gly Leu Ser Arg Ala Ala Tyr Pro  
 195 200 205

Pro Glu Leu Arg Phe Met Tyr Tyr Val Asp Gly Arg Gly Lys Asp Gly  
 210 215 220

Gly Phe Tyr Gln Val Lys Glu Ala Val Ile His Tyr Leu Glu Thr Leu  
 225 230 235 240

Ser

<210> 5  
 <211> 39  
 <212> DNA  
 <213> Homo sapiens

<220>  
 <221> misc\_feature  
 <223> naturally occurring TIRAP inhibitory peptide

<400> 5  
 ctgcaactcc gggatgcaac cccaggcgcc gctatagtg 39

<210> 6  
 <211> 42  
 <212> DNA  
 <213> Homo sapiens

<220>  
 <221> misc\_feature  
 <223> degenerate TIRAP polynucleotide

<220>  
 <221> misc\_feature  
 <222> (1)..(42)  
 <223> Y=C,T; N=A,G,C,T; R=A,G; H=A,C,T; W=A,T; S=G,C

<400> 6  
 ytnccarytnm gngaygcac nccngnggn gcnathgtnw sn 42

<210> 7  
 <211> 42  
 <212> DNA

<213> Mus sp.

<220>

<221> misc\_feature

<223> naturally occurring TIRAP inhibitory peptide

<400> 7

ctgcagcttc gggatgcagc cccgggtggc gccattgttt cg

42

<210> 8

<211> 42

<212> DNA

<213> Mus sp.

<220>

<221> misc\_feature

<223> degenerate TIRAP polynucleotide

<220>

<221> misc\_feature

<222> (1)..(42)

<223> Y=C,T; N=A,G,C,T; R=A,G; M=A,C; H=A,C,T; W=A,T; S=G,C

<400> 8

ytnccarytnm gngaygcngc nccnggnggn gcnathgtnw sn

42

<210> 9

<211> 14

<212> PRT

<213> Homo sapiens

<220>

<221> MISC\_FEATURE

<223> TIRAP inhibitory polypeptide

<400> 9

Leu Gln Leu Arg Asp Ala Thr Pro Gly Gly Ala Ile Val Ser

1

5

10

<210> 10

<211> 14

<212> PRT

<213> Mus sp.

<220>

<221> MISC\_FEATURE

<223> TIRAP inhibitory polypeptide

<400> 10

Leu Gln Leu Arg Asp Ala Ala Pro Gly Gly Ala Ile Val Ser

1

5

10

<210> 11  
<211> 14  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> TIRAP inhibitory peptide  
<220>  
<221> MISC\_FEATURE  
<223> TIRAP inhibitory polypeptide

<400> 11  
Val Ser Asp Arg Asp Val Leu Pro Gly Thr Cys Val Trp Ser  
1 5 10

<210> 12  
<211> 30  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> TIRAP/Antennapedia fusion protein  
<220>  
<221> MISC\_FEATURE  
<223> TIRAP/Antennapedia fusion protein

<400> 12  
Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys  
1 5 10 15

Leu Gln Leu Arg Asp Ala Ala Pro Gly Gly Ala Ile Val Ser  
20 25 30